ENDOGLUCANASES

FIELD OF THE INVENTION

The present invention relates to novel enzyme preparations comprising an enzyme exhibiting endoglucanase activity which performs very good in industrial applications such as laundry compositions, for biopolishing of newly manufactured textiles, for providing an abraded look of cellulosic fabric or garment, and for treatment of paper pulp. Further, the invention relates to DNA constructs encoding such enzymes, a method for providing a gene encoding for such enzymes, a method of producing the enzymes, enzyme preparations containing such enzymes, and the use of these enzymes for a number of industrial applications.

BACKGROUND OF THE INVENTION

Cellulases or cellulytic enzymes are enzymes involved in hydrolysis of cellulose. In the hydrolysis of native cellulose, it is known that there are three major types of cellulase enzymes involved, namely cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91), endo-β-1,4-glucanase (endo-1,4-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21).

Cellulases are synthesized by a large number of microorganisms which include fungi, actinomycetes, myxobacteria and true bacteria but also by plants. Especially endoglucanases of a wide variety of specificities have been identified.

A very important industrial use of cellulytic enzymes is the use for treatment of cellulosic textile or fabric, e.g. as ingredients in detergent compositions or fabric softener compositions, for bio-polishing of new fabric (garment finishing), and for obtaining a "stone-washed" look of cellulose-containing fabric, especially denim, and several methods for such treatment have been suggested, e.g. in GB-A-1 368 599, EP-A-0 307 564 and EP-A-0 435 876, WO 91/17243, WO 91/10732, WO 91/17244, PCT/DK95/000108 and PCT/DK95/00132.

Another important industrial use of cellulytic enzymes is the use for treatment of paper pulp, e.g. for improving the drainage or for deinking of recycled paper.

Especially the endoglucanases (EC No. 3.2.1.4) constitute an interesting group of hydrolases for the mentioned industrial uses. Endoglucanases catalyses endo hydrolysis of 1,4-β-D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxy methyl cellulose and hydroxy ethyl cellulose), lichenin, β-1,4 bonds in mixed β-1,3 glucans such as cereal β-D-glucans or xyloglucans and other plant material containing cellulosic parts. The authorized name is endo-1,4-β-D-glucan 4-glucano hydrolase, but the abbreviated term endoglucanase is used in the present specification. Reference can be made to T.-M. Enveri, "Microbial Cellulases" in W.M. Fogarty, Microbial Enzymes

and Biotechnology, Applied Science Publishers, p. 183-224 (1983); Methods in Enzymology, (1988) cVol. 160, p. 200-391 (edited by Wood, W.A. and Kellogg, S.T.); Béguin, P., "Molecular Biology of Cellulose Degradation", Annu. Rev. Microbiol. (1990), Vol. 44, pp. 219-248; Béguin, P. and Aubert, J-P., "The biological degradation of cellulose", FEMS Microbiology Reviews 13 (1994) p.25-58; Henrissat, B., "Cellulases and their interaction with cellulose", Cellulose (1994), Vol. 1, pp. 169-196.

Fungal endoglucanases have been described in numerous publications, especially those derived from species as e.g. *Fusarium oxysporum*, *Trichoderma reesei*, *Trichoderma longibrachiatum*, *Aspergillus aculeatus*, *Neocallimastix patriciarum*, and e.g. from species of the genera *Piromyces*, *Humicola, Myceliophthora, Geotricum, Penicillium, Irpex, Coprinus.*

For example, fungal endoglucanases have been described by Sheppard, P.O., et al., "The use of conserved cellulase family-specific sequences to clone Cellulase homologue cDNAs from *Fusarium oxysporum*, Gene, (1994), Vol. 15, pp. 163-167, Saloheimo, A., et al., "A novel, small endoglucanase gene, *egl5*, from *Trichoderma reesei* isolated by expression in yeast", Molecular Microbiology (1994), Vol. 13(2), pp. 219-228; van Arsdell, J.N. et al., (1987), Cloning, characterization, and expression in *Saccharomyces cerevisiae* of endoglucanase I from *Trichoderma reesei*, Bio/Technology **5**: 60-64; Penttilä, M. et al., (1986), "Homology between cellulase genes of *Trichoderma reesei*: complete nucleotide sequence of the endoglucanase I gene", *Gene* **45**:253-263; Saloheimo, M. et al, (1988), "EGIII, a new endoglucanase from *Trichoderma reesei*: the characterization of both gene and enzyme", *Gene* **63**:11-21; Gonzáles, R., et al., "Cloning, sequence analysis and yeast expression of the *egl1* gene from *Trichoderma longibrachiatum*", Appl. Microbiol. Biotechnol., (1992), Vol. 38, pp. 370-375; Ooi, T. et al. "Cloning and sequence analysis of a cDNA for cellulase (FI-CMCase) from *Aspergillus aculeatus*", Curr. Genet., (1990), Vol. 18, pp. 217-222; Ooi, T. et al, "Complete nucleotide sequence of a gene coding for *Aspergillus aculeatus* cellulase (FI-CMCase)", Nucleic Acids Research, (1990), Vol. 18, No. 19, p. 5884; Xue, G. et al., "Cloning and expression of multiple cellulase cDNAs from the anaerobic rumen fungus *Neocallimastix patriciarum* in *E. coli*", J. Gen. Microbiol., (1992), Vol. 138, pp. 1413-1420; Xue, G. et al., "A novel polysaccharide hydrolase cDNA (*celD*) from *Neocallimastix patriciarum* encoding three multi-functional catalytical domains with high endoglucanase, cellobiohydrolase and xylanase activities", J. Gen. Microbiol., (1992), Vol. 138, pp. 2397-2403; Zhou, L. et al., "Intronless *celB* from the anaerobic fungus *Neocallimastix patriciarum* encodes a modular family A endoglucanase", Biochem. J., (1994), Vol. 297, pp. 359-364; Dalbøge, H. and Heldt-Hansen, H.P., "A novel method for efficient expression cloning of fungal enzyme genes", Mol. Gen. Genet., (1994), Vol. 243, pp. 253-260; Ali, B.R.S. et al., "Cellulases and hemicellulases of the anaerobic fungus *Piromyces* constitute a multiprotein cellulose-binding complex and are encoded by multigene

families", FEMS Microbiol. Lett., (1995), Vol. 125, No. 1, pp. 15-21. Further, the DNA Data Bank of Japan (DDBJ database publicly available at Internet) comprises two DNA sequences cloned from *Penicillium janthinellum* encoding endoglucanases (cloned by A. Koch and G. Memitz, respectively) and a DNA sequence cloned from *Humicola grisea var. thermoidea* encoding an endoglucanase (cloned by T. Uozumi). Two endoglucanases from *Macrophomina phaseolina* have been cloned and sequenced, see Wang, H.Y. and Jones, R.W.: "Cloning, characterization and functional expression of an endoglucanase-encoding gene from the phytopathogenic fungus *Macrophomina phaseolina*" in Gene, 158:125-128, 1995, and Wang, H.Y. and Jones, R.W.: "A unique endoglucanase-encoding gene cloned from the phytopathogenic fungus *Macrophomina phaseolina*" in Applied And Environmental Microbiology, 61:2004-2006, 1995. One of these endoglucanases shows high homology to the egl3 endoglucanase from the fungus *Trichoderma reesei*, the other shows homology to the egl1 from the microbial phytopathogen *Pseudomonas solanacearum* indicating that both endoglucanases belong to family 5 of glycosyl hydrolases (B. Henrissat, Biochem J **280**:309-316 (1991)). Filament-specific expression of a cellulase gene in the dimorphic fungus *Ustilago maydis* is disclosed in Schauwecker, F. et al. (1995).

WO 91/17243 (Novo Nordisk A/S) discloses a cellulase preparation consisting of a homogenous endoglucanase component immunoreactive with an antibody raised against a highly purified 43 kDa endoglucanase derived from *Humicola insolens*, DSM 1800; WO 91/17244 (Novo Nordisk A/S) discloses a new (hemi)cellulose degrading enzyme, such as an endoglucanase, a cellobiohydrolase or a β-glucosidase, which may be derived from fungi other than *Trichoderma* and *Phanerochaete*; WO 93/20193 discloses an endoglucanase derivable from *Aspergillus aculeatus*; WO 94/21801 (Genencor Inc.) concerns a cellulase system isolated from *Trichoderma longibrachiatum* exhibiting endoglucanase activity; WO 94/26880 (Gist Brocades N.V.) discloses an isolated mixture of cellulose degrading enzymes, which preferable are obtained from *Trichoderma*, *Aspergillus* or *Disporotrichum*, comprising endoglucanase, cellobiohydrolase, and xyloglucanase activity; and WO 95/02043 (Novo Nordisk A/S) describes an enzyme with endoglucanase activity derived from *Trichoderma harzianum*, which can be used for a number of purposes including *e.g.* degradation or modification of plant cell walls.

It is also known that cellulases may or may not have a cellulose binding domain (a CBD). The CBD enhances the binding of the enzyme to a cellulose-containing fiber and increases the efficacy of the catalytic active part of the enzyme.

There is an ever existing need for providing novel cellulase enzyme preparations which may be used for applications where cellulase, preferably an endoglucanase, activity is desirable.

The object of the present invention is to provide novel enzyme preparations having substantial cellulytic activity at acid, neutral or alkaline conditions and improved performance in paper pulp processing, textile treatment, laundry processes or in animal feed; preferably novel cellulases, more preferably well-performing endoglucanases, which are contemplated to be producible or produced by recombinant techniques.

SUMMARY OF THE INVENTION

Surprisingly, it has been found that a group of endoglucanases having certain unique characteristics perform very good in those industrial applications for which endoglucanases are conventionally used. These unique characteristics can be described in terms of conserved regions of the amino acid sequence of the enzyme protein and the inventors have found that cellulytic enzymes, i.e. enzymes exhibiting cellulytic activity, having certain conserved regions are very effective e.g. in the treatment of laundry, in the treatment of newly manufactured textile, in the treatment of papermaking pulp.

Accordingly, in its first aspect the present invention relates to an enzyme preparation consisting essentially of an enzyme having cellulytic activity and comprising a first amino acid sequence consisting of 14 amino acid residues having the following sequence

Thr	Arg	Xaa	Xaa	Asp	Cys	Cys	Xaa	Xaa	Xaa	Cys	Xaa	Trp	Xaa
1	2	3	4	5	6	7	8	9	10	11	12	13	14

(SEQ ID NO: 79)

and a second amino acid sequence consisting of 5 amino acid residues having the following sequence

Trp	Cys	Cys	Xaa	Cys
1	2	3	4	5

(SEQ ID NO: 80)

wherein,

at position 3 of the first sequence, the amino acid is Trp, Tyr or Phe;

at position 4 of the first sequence, the amino acid is Trp, Tyr or Phe;

at position 8 of the first sequence, the amino acid is Arg, Lys or His;

at positions 9, 10, 12 and 14, respectively, of the first sequence, and at position 4 of the second sequence, the amino acid is any of the 20 naturally occurring amino acid residues with the provisos that, in the first amino acid sequence, (i) when the amino residue at position 12 is Ser, then the amino acid residue at position 14 is not Ser, and (ii) when the amino residue at position 12 is Gly, then the amino acid residue at position 14 is not Ala.

This surprising finding of clearly recognisable conserved regions, in spite of rather prominent variations found within well-performing endoglucanase enzymes, is a result of studies of a number

of fungal DNA sequences encoding for specific amino acid sequences of enzymes having significant cellulytic, especially endoglucanase, activities.

Based on this finding, a novel molecular method taylored to screen specifically for genomic DNA or cDNA characterised by encoding the enzymes of the invention has been developed. As tools for this three sets of degenerated primers were constructed. Accordingly, in its second aspect, the invention relates to a method for providing a gene encoding for cellulytic enzymes having the above conserved regions.

By using this method, i.e. the set of primers for a PCR screening on genomic DNA, it was surprisingly found that DNA encoding for said enzymes can be found from a broad range of fungi, belonging to taxonomically very different organisms and inhabiting ecologically very different niches.

Further, by using this method it has been possible to find DNA sequences encoding for the core regions (catalytically active regions or domains) of said enzymes without any attached cellulose binding domain (CBD) which core regions of enzymes would not have been selected by using conventional performance based screening approaches. The inventors have verified experimentally that the linking of a CBD region to a core region enzyme (comprising the catalytically active region or domain of the enzyme) of the present invention results in a significantly improved performance, e.g. a fifty times higher performance, of the multiple domain enzyme.

Accordingly, the present invention provides novel cellulases, especially endoglucanases, having improved performance in industial applications, either in their native form, or homo- or heterologously produced.

In further aspects, the present invention relates to novel cellulytic enzyme preparations which are derivable from taxonomically specific phyli, classes, orders, families, genera, and species; e.g. from Basidiomycotous *Hymenomycetes*, *Zygomycota*, *Chytridiomycota;* or from the classes *Discomycetes, Loculoascomycetes, Plectomycetes*; *Archaeascomycetes, Hemiascomycetes* or from the orders *Diaportales, Xylariales, Trichosphaeriales, Phyllachorales*; or from the families *Nectriaeae, Sordariaceae, Chaetomiaceae, Ceratostomaceae, Lasiosphaeriaceae*; or from the genera *Cylindrocarpon, Gliocladium, Volutella, Scytalidium, Acremonium*, or from the species *Fusarium lycopersici, Fusarium passiflora, Fusarium solani, Fusarium anguioides, Fusarium poae, Humicola nigrescens*, *Humicola grisea*, especially such consisting essentially of an enzyme comprising an amino acid sequence selected from the group consisting of the sequences (SEQ ID NOS: 105-107)

Xaa Thr Arg Xaa Phe Asp Xaa
1 2 3 4 5 6 7;
Xaa Thr Arg Xaa Tyr Asp Xaa
1 2 3 4 5 6 7; and

Xaa Thr Arg Xaa Trp Asp Xaa
1 2 3 4 5 6 7

wherein, at position 4, Xaa is Trp, Tyr or Phe; and at positions 1 and 7, Xaa is any of the 20 naturally occurring amino acid residues.

More specifically, the enzyme preparation of the invention is preferably obtainable from the taxonomically specific phyli, classes, orders, families, genera, and species mentioned above which all produce endoglucanases comprising a first peptide consisting of 13 amino acid residues having the following sequence

Thr Arg Xaa Xaa Asp Cys Cys Xaa Xaa Xaa Cys Xaa Trp (SEQ ID NO: 108)
1 2 3 4 5 6 7 8 9 10 11 12 13

and a second peptide consisting of 5 amino acid residues having the following sequence

Trp Cys Cys Xaa Cys (SEQ ID NO: 80)
1 2 3 4 5

wherein, at position 3 of the first sequence, the amino acid is Trp, Tyr or Phe; at position 4 of the first sequence, the amino acid is Trp, Tyr or Phe; at position 8 of the first sequence, the amino acid is Arg, Lys or His; at positions 9, 10, and 12, respectively, of the first sequence, and at position 4 of the second sequence, the amino acid is any of the 20 naturally occurring amino acid residues.

In yet further aspects, the present invention provides DNA constructs comprising a DNA sequence encoding an enzyme exhibiting endoglucanase activity, which DNA sequence comprises the DNA sequence of SEQ ID NOS: 1, 7, 9, 11, 13, 15, 21, and 25, respectively, or analogues thereof.

The present invention also relates to a recombinant expression vector comprising a DNA construct of the invention; to a cell comprising a DNA construct or a recombinant expression vector of the invention; to a method of producing an enzyme, e.g a recombinant enzyme, of the invention; to a method of providing colour clarification of laundry by using the enzyme of the invention; to a laundry composition comprising the enzyme of the invention; to uses of the enzyme of the invention for degradation or modification of plant material, e.g. cell walls, for treatment of fabric, textile or garment, for treatment of paper pulp; and to an enzyme preparation which is enriched in an enzyme of the present invention.

THE DRAWINGS

Figure 1 is an alignment of the deduced encoded amino acid sequences of *Acremonium sp. (I)*, *Volutella colletotrichoides*, *Crinipellis scabella*, *Acremonium sp. (II)*, *Myceliophthora thermophila*, *Thielavia terrestris*, *Macrophomina phaseolina*. The Pileup program (Feng and Doolittle, 1987)

(GCG package, version 8.0) was used to create the best alignment. Identical residues in at least four sequences (boxed) are indicated around the corresponding amino acids.

Figure 2a, b, c illustrates the taxonomic classification within the Fungal Kingdom of all the microorganisms disclosed herein as being capable of producing said enzyme preparations and enzymes of the invention.

The taxonomic classification used herein builds primarily on the system used in the NIH Data Base (Entrez, version spring 1996) available on World Wide Web: (http://www3.ncbi.nlm.nih.gov/htbin/ef/entrezTAX).

Regarding classification of organisms which are not included in the Entrez data base the following generally available and world wide accepted reference books have been used:

For *Ascomycetes*: Eriksson, O.E. & Hawksworth, D.L.: Systema Ascomycetum vol 12 (1993).

For *Basidiomycetes*: Jülich, W.: Higher Taxa of Basidiomycetes, Bibliotheca Mycologia 85, 485pp (1981).

For *Zygomycetes*: O´Donnell, K.: Zygomycetes in culture, University of Georgia, US, 257pp (1979).

General mycological reference books:

Hawksworth, D.L., Kirk, P.M., Sutton, B.C. and Pegler, D.N.: Dictionary of the fungi, International Mycological Institute, 616pp (1995);

Von Arx, J.A.: The genera of fungi sporulating in culture, 424pp (1981).

The taxonomic implacement of the genus Humicola has untill recently remained unclear. However, studies of 18SRNA of a wide selection of Sordariales has given strong indications of referring Humicola to the order Sordariales (Taylor, Clausen & Oxenbøll, unpublished). Further these data suggests Humicola along with Scytalidium to be only rather distantly related to the families Sordariaceae, Chaetomiaceae, Ceratostomataceae, and Lasiosphaeriaceae. In accordance with the above Humicola and Scytalidium are here placed within the order Sordariales, with unclassified Family.

Figure 3 is an alignment of the deduced partial amino acid sequences derived from a selection of 26 of the 46 microorganisms described in Example 5.

DETAILED DESCRIPTION OF THE INVENTION

In the present context, the term "the 20 naturally occuring amino acid residues" denotes the 20 amino acid residues usually found in proteins and conventionally known as alanine (Ala or A), valine (Val or V), leucine (Leu or L), isoleucine (Ile or I), proline (Pro or P), phenylalanine (Phe or F), tryptophan (Trp or W), methionine (Met or M), glycine (Gly or G), serine (Ser or S), threonine (Thr or

T), cysteine (Cys or C), tyrosine (Tyr or Y), asparagine (Asn or N), glutamine (Gln or Q), aspartic acid (Asp or D), glutamic acid (Glu or E), lysine (Lys or K), arginine (Arg or R), and histidine (His or H).

According to the present invention there is provided novel well-performing endoglucanases comprising conserved amino acid sequence regions, especially a first amino acid sequence consisting of 14 amino acid residues having the following sequence

Thr Arg Xaa Xaa Asp Cys Cys Xaa Xaa Xaa Cys Xaa Trp Xaa (SEQ ID NO: 79)
1 2 3 4 5 6 7 8 9 10 11 12 13 14

and a second amino acid sequence consisting of 5 amino acid residues having the following sequence

Trp Cys Cys Xaa Cys (SEQ ID NO: 80)
1 2 3 4 5

wherein,

at position 3 of the first sequence, the amino acid is Trp, Tyr or Phe;

at position 4 of the first sequence, the amino acid is Trp, Tyr or Phe;

at position 8 of the first sequence, the amino acid is Arg, Lys or His;

at positions 9, 10, 12 and 14, respectively, of the first sequence, and at position 4 of the second sequence, the amino acid is any of the 20 naturally occurring amino acid residues with the provisos that, in the first amino acid sequence, (i) when the amino residue at position 12 is Ser, then the amino acid residue at position 14 is not Ser, and (ii) when the amino residue at position 12 is Gly, then the amino acid residue at position 14 is not Ala.

Preferably, the enzyme of the invention is of microbial origin, i.e. obtainable from a microorganism such as a fungus.

In a preferred embodiment, the amino acid residue at position 9 of the first sequence is selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, cysteine, asparagine, glutamine, tyrosine, serine, methionine and tryptophan, preferably from the group consisting of proline and threonine.

In another preferred embodiment, the amino acid residue at position 10 of the first sequence is selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, cysteine, asparagine, glutamine, tyrosine, serine, methionine and tryptophan, preferably serine.

In yet another preferred embodiment, the amino acid residue at position 12 of the first sequence is selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, cysteine, asparagine, glutamine, tyrosine, serine, methionine and tryptophan, preferably from the group consisting of alanine and glycine.

In yet another preferred embodiment, the amino acid residue at position 14 of the first sequence is selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, cysteine, asparagine, glutamine, tyrosine, serine, methionine, tryptophan, glutamic acid and aspartic acid, preferably from the group consisting of proline, threonine, serine, alanine, glutamic acid and aspartic acid.

Preferably, the amino acid residue at position 4 of the second sequence is selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, cysteine, asparagine, glutamine, tyrosine, serine, methionine, tryptophan, glutamic acid and aspartic acid, more preferably from the group consisting of alanine, glycine, and glutamine.

Examples of more preferred embodiments are such wherein, in the first sequence, the amino acid residue at position 3 is tyrosine; or the amino acid residue at position 4 is tryptophan; or the amino acid residue at position 8 is lysine.

In an especially preferred embodiment, the enzyme of the invention has a first sequence comprising the amino acid sequence

Thr Arg Tyr Trp Asp Cys Cys Lys Pro Ser Cys Ala Trp (SEQ ID NO: 79)
1 2 3 4 5 6 7 8 9 10 11 12 13,

or the amino acid sequence

Thr Arg Tyr Trp Asp Cys Cys Lys Thr Ser Cys Ala Trp (SEQ ID NO: 79)
1 2 3 4 5 6 7 8 9 10 11 12 13,

or the amino acid sequence

Thr Arg Tyr Trp Asp Cys Cys Lys Pro Ser Cys Gly Trp (SEQ ID NO: 79)
1 2 3 4 5 6 7 8 9 10 11 12 13.

In a second aspect, the present invention provides a method for providing a microbial strain comprising a gene encoding such an enzyme which method comprises hybridization, e.g. PCR amplification, under standard conditions with an oligonucleotide derived from any of the conserved regions, illustrated in Fig.1.

A useful oligonucleotide comprises a nucleotide sequence encoding at least a pentapeptide comprised in a peptide selected from the group consisting of

a.

Thr Arg Xaa Xaa Asp Cys Cys Xaa Xaa Xaa Cys Xaa Trp Xaa (SEQ ID NO: 79)
1 2 3 4 5 6 7 8 9 10 11 12 13 14

the amino acid at position 3 or 4 being Trp, Tyr or Phe;

the amino acid in position 8 being Arg, Lys or His;

the amino acid at positions 9, 10, 12 and 14, respectively, being any of the 20 naturally occurring amino acid residues ; and

b.

Trp Cys Cys Xaa Cys Tyr (SEQ ID NO: 81)

1 2 3 4 5 6

the amino acid at position 4 being any of the 20 naturally occurring amino acid residues ; and

c.

Xaa Pro Gly Gly Gly Xaa Gly Xaa Phe (SEQ ID NO: 82)

1 2 3 4 5 6 7 8 9

the amino acid at position 1 being Met or Ile;

the amino acid at positions 6 and 8, respectively, is Leu, Ile or Val; and

d.

Gly Cys Xaa Xaa Arg Xaa Asp Trp Xaa (SEQ ID NO: 83)

1 2 3 4 5 6 7 8 9

the amino acid at position 3 being any of the 20 naturally occurring amino acid residues;

the amino acid at positions 4 and 6, respectively, being Trp, Tyr or Phe; and

the amino acid at position 9 being Phe or Met;

The useful oligonucleotides also comprises nucleotide sequences complementary to the sequences mentioned.

In a preferred embodiment of the method of the invention, the oligonucleotide corresponds to a PCR primer selected from the PCR primers

sense:

5'-CCCCAAGCTTACI$^{A}/_{C}$GITA$^{C}/_{T}$TGGGA$^{C}/_{T}$TG$^{C}/_{T}$TG$^{C}/_{T}$AA$^{A}/_{G}$$^{A}/_{C}$C-3' (SEQ ID NO: 84)

antisense 1:

5'- CTAGTCTAGATA$^{A}/_{G}$CAIGC$^{A}/_{G}$CA$^{A}/_{G}$CACC -3' (SEQ ID NO: 85);

antisense 2:

CTAGTCTAGAAAIA$^{A}/_{G}/^{T}$ICCIA$^{A}/^{C}/^{G}$ICCICCICCIGG -3' (SEQ ID NO: 86);

antisense 3:

5'- CTAGTCTAGAIAACCA$^{A}/_{G}$TCA$^{A}/_{G}$$^{A}/_{T}AIC^{G}/_{T}$CC-3 (SEQ ID NO: 87).

In a third aspect, the present invention provides an enzyme preparation which essentially consists of an enzyme having cellulytic activity and having the conserved regions found by the inventors, i.e. which comprises a peptide consisting of 7 amino acid residues having the following sequence (SEQ ID NOS: 105-107)

Xaa Thr Arg Xaa Phe Asp Xaa

1 2 3 4 5 6 7;

Xaa Thr Arg Xaa Tyr Asp Xaa

1 2 3 4 5 6 7; and

Xaa Thr Arg Xaa Trp Asp Xaa

1 2 3 4 5 6 7

wherein, at position 4, Xaa is Trp, Tyr or Phe; and

at positions 1 and 7, Xaa is any of the 20 naturally occurring amino acid residues.

This enzyme is obtainable from a strain belonging to Basidiomycotous *Hymenomycetes* (see Fig. 2), more preferably to the group consisting of the orders *Agaricales, Auriculariales,* and *Aphyllophorales*, even more preferably to the group consisting of the families *Exidiaceae, Tricholomataceae, Coprinaceae, Schizophyllaceae, Bjerkanderaceae* and *Polyporaceae*, especially to the group consisting of the genera *Exidia, Crinipellis, Fomes, Panaeolus, Trametes, Schizophyllum,* and *Spongipellis*.

Specific examples are endoglucanases obtainable from a strain belonging to the group consisting of the species *Exidia glandulosa, Crinipellis scabella, Fomes fomentarius,* and *Spongipellis sp.*, more specific examples being *Exidia glandulosa,* CBS 277.96, *Crinipellis scabella,* CBS 280.96, *Fomes fomentarius,* CBS 276.96, and *Spongipellis sp.*, CBS 283.96.

Exidia glandulosa was deposited at Centraalbureau voor Schimmelcultures, Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, the Netherlands, on 12 March, 1996, under the deposition number CBS 277.96; *Crinipellis scabella* was deposited at Centraalbureau voor Schimmelcultures on 12 March, 1996, under the deposition number CBS 280.96, *Fomes fomentarius* was deposited at Centraalbureau voor Schimmelcultures on 12 March, 1996, under the deposition number CBS 276.96, and *Spongipellis sp.* was deposited at Centraalbureau voor Schimmelcultures on 12 March, 1996, under the deposition number CBS 283.96; all deposited under the Budapest Treaty.

The enzyme preparation of the invention is also obtainable from a strain belonging to *Chytridiomycota*, preferably from a strain belonging to the class of *Chytridiomycetes*, more preferably belonging to the group consisting of the order *Spizellomycetales*, even more preferably to the family *Spizellomycetaceae*, especially belonging to the genus *Rhizophlyctis.* A specific example is a strain belonging to the species *Rhizophlyctis rosea*, more specifically to *Rhizophlyctis rosea*, CBS 282.96.

Rhizophlyctis rosea was deposited at Centraalbureau voor Schimmelcultures on 12 March 1996, under the deposition number CBS 282.96; under the Budapest Treaty.

The enzyme preparation of the invention is also obtainable from a strain belonging to *Zygomycota*, preferably belonging to the class *Zygomycetes*, more preferably to the order *Mucorales,* even more preferably to the group of families consisting of *Mucoraceae* and *Thamnidiaceae*, especially belonging to the group consisting of the genera *Rhizomucor,*

Phycomyces and Chaetostylum. Specific examples are strains belonging to the genera *Rhizomucor pusillus, Phycomyces nitens*, and *Chaetostylum fresenii* more specifically to *Rhizomucor pusillus,* IFO 4578, and *Phycomyces nitens*, IFO 4814 and *Chaetostylum fresenii*, NRRL 2305.

Further, the enzyme preparation of the invention is also obtainable from a strain belonging to the group consisting of *Archaeascomycetes, Discomycetes, Hemiascomycetes, Loculoascomycetes, and Plectomycetes,* preferably belonging to the group consisting of the orders *Pezizales, Rhytismatales, Dothideales,* and *Eurotiales.* Especially, the enzyme is obtainable from a strain belonging the the group consisting of the families *Cucurbitariaceae, Ascobolaceae, Rhytismataceae,* and *Trichocomaceae,* preferably belonging the the group consisting of the genera *Diplodia, Microsphaeropsis, Ulospora, Macrophomina, Ascobolus, Saccobolus, Penicillium,* and *Thermomyces.* Specific examples are enzymes obtainable from a strain belonging the the group consisting of the species *Diplodia gossypina, Microsphaeropsis sp., Ulospora bilgramii, Aureobasidium sp., Macrophomina phaseolina, Ascobolus stictoides, Saccobolus dilutellus, Peziza, Penicillium verruculosum, Penicillium chrysogenum,* and *Thermomyces verrucosus;* more specifically *Diplodia gossypina, CBS 274.96, Ulospora bilgramii, NKBC 1444, Macrophomina phaseolina, CBS 281.96, Saccobolus dilutellus, CBS 275.96, Penicillium verruculosum, ATCC 62396, Penicillium chrysogenum,* ATCC 9480, and *Thermomyces verrucosus*, CBS 285.96.

Diplodia gossypina was deposited at Centraalbureau voor Schimmelcultures on 12 March, 1996, under the deposition number CBS 274.96, *Macrophomina phaseolina* was deposited at Centraalbureau voor Schimmelcultures on 12 March, 1996, under the deposition number CBS 281.96, *Saccobolus dilutellus* was deposited at Centraalbureau voor Schimmelcultures on 12 March, 1996, under the deposition number CBS 275.96; *Thermomyces verrucosus* was deposited at Centraalbureau voor Schimmelcultures on 12 March, 1996, under the deposition number CBS 285.96; all under the Budapest Treaty.

Yet further, the enzyme is obtainable from a strain belonging to the group consisting of the orders *Diaportales, Xylariales, Trichosphaeriales* and *Phyllachorales,* preferably from a strain belonging to the group consisting of the families *Xylariaceae, Valsaceae,* and *Phyllachoraceae*, more preferably belonging to the genera *Diaporthe, Colletotrichum, Nigrospora, Xylaria, Nodulisporum* and *Poronia.* Specific examples are the species *Diaporthe syngenesia, Colletotrichum lagenarium, Xylaria hypoxylon, Nigrospora sp., Nodulisporum sp.*, and *Poronia punctata,* more specifically *Diaporthe syngenesia, CBS 278.96, Colletotrichum lagenarium, ATCC 52609, Nigrospora sp., CBS 272.96, Xylaria hypoxylon, CBS 284.96.*

Diaporthe syngenesia was deposited at Centraalbureau voor Schimmelcultures on 12 March, 1996, under the deposition number CBS 278.96, *Nigrospora sp.* was deposited at

Centraalbureau voor Schimmelcultures on 12 March, 1996, under the deposition number CBS 272.96, *Xylaria hypoxylon* was deposited at Centraalbureau voor Schimmelcultures on 12 March, 1996, under the deposition number CBS 284.96; all under the Budapest Treaty.

The enzyme is also obtainable from the unidentified fungal, mitosporic, coleomycetous deposited at Centraalbureau voor Schimmelcultures on 12 March 1996, under the deposition numbers CBS 270.96, CBS 271.96 and CBS 273.96, respectively, under the Budapest Treaty.

The enzyme is also obtainable from a strain belonging to the group consisting of the genera *Cylindrocarpon, Gliocladium, Nectria, Volutella, Sordaria, Scytalidium, Thielavia, Syspastospora, Cladorrhinum, Chaetomium, Myceliphthora* and *Acremonium*, especially from a strain belonging to the group consisting of the species *Cylindrocarpon sp., Nectria pinea, Volutella colletotrichoides, Sordaria fimicola, Sordaria macrospora, Thielavia terrestris, Thielavia thermophila, Syspastospora boninensis, Cladorrhinum foecundissimum, Chaetomium murorum, Chaetomium virescens, Chaetomium brasiliensis, Chaetomium cunicolorum, Myceliophthora thermophila, Gliocladium catenulatum, Scytalidium thermophila,* and *Acremonium sp.*, more specifically from *Nectria pinea*, CBS 279.96, *Volutella colletotrichoides*, CBS 400.58, *Sordaria fimicola*, ATCC 52644, *Sordaria macrospora*, ATCC 60255, *Thielavia terrestris*, NRRL 8126, *Thielavia thermophila*, CCBS 174.70, *Chaetomium murorum*, CBS 163.52, *Chaetomium virescens*, CBS 547.75, *Chaetomium brasiliensis*, CBS 122.65, *Chaetomium cunicolorum*, CBS 799.83, *Syspastospora boninensis*, NKBC 1515, *Cladorrhinum foecundissimum*, ATCC 62373, *Myceliophthora thermophila,* CBS 117.65, *Scytalidium thermophila,* ATCC 28085, Gliocladium catenulatum, ATCC 10523, and *Acremonium sp.*, CBS 478.94.

Nectria pinea was deposited at Centraalbureau voor Schimmelcultures on 12 March, 1996, under the deposition number CBS 279.96, and *Acremonium sp.* was deposited on 28 September 1994 under the deposition number CBS 478.94, both according to the Budapest Treaty.

The enzyme is also obtainable from a strain belonging to the group consisting of the species *Fusarium solani, Fusarium anguioides, Fusarium poae, Fusarium oxysporum ssp. lycopersici, Fusarium oxysporum ssp. passiflora, Humicola nigrescens* and *Humicola grisea*, especially *Fusarium oxysporum ssp lycopersici, CBS 645.78, Fusarium oxysporum ssp passiflora, CBS 744.79, Fusarium solani, IMI 107.511, Fusarium anguioides, IFO 4467, Fusarium poae, ATCC 60883, Humicola nigrescens*, CBS 819.73 and *Humicola grisea*, ATCC 22726. It is to be noted that *Humicola grisea* is different from *Humicola grisea var. thermoidea*.

In a preferred embodiment, the enzyme preparation of the invention is derived from the disclosed classes, orders, families, genera and species and essentially consists of an enzyme comprising a first peptide consisting of 13 amino acid residues having the following sequence

Thr Arg Xaa Xaa Asp Cys Cys Xaa Xaa Xaa Cys Xaa Trp (SEQ ID NO: 79)
1 2 3 4 5 6 7 8 9 10 11 12 13

and a second peptide consisting of 5 amino acid residues having the following sequence

Trp Cys Cys Xaa Cys (SEQ ID NO: 80)
1 2 3 4 5

wherein, at position 3 of the first sequence, the amino acid is Trp, Tyr or Phe; at position 4 of the first sequence, the amino acid is Trp, Tyr or Phe; at position 8 of the first sequence, the amino acid is Arg, Lys or His; at positions 9, 10, and 12, respectively, of the first sequence, and at position 4 of the second sequence, the amino acid is any of the 20 naturally occurring amino acid residues.

Preferably, the amino acid residue at position 9 of the first sequence is selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, cysteine, asparagine, glutamine, tyrosine, serine, methionine and tryptophan, more preferably from the group consisting of proline and threonine; the amino acid residue at position 10 of the first sequence which is selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, cysteine, asparagine, glutamine, tyrosine, serine, methionine and tryptophan, preferably serine; the amino acid residue at position 12 of the first sequence is selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, cysteine, asparagine, glutamine, tyrosine, serine, methionine and tryptophan, preferably from the group consisting of alanine and glycine; and the amino acid residue at position 4 of the second sequence is selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, cysteine, asparagine, glutamine, tyrosine, serine, methionine, tryptophan, glutamic acid and aspartic acid, more preferably from the group consisting of alanine, glycine, and glutamine.

In further aspects, the present invention provides a DNA construct comprising a DNA sequence encoding an enzyme exhibiting endoglucanase activity, which DNA sequence comprises

a) the DNA sequence of SEQ ID NO: 1, 7, 9, 11, 13, 15, 21, or 25, respectively, or the DNA sequence obtainable from the plasmid in *Saccharomyces cerevisiae* DSM 9770, DSM 10082, DSM 10080, DSM 10081, *Escherichia coli*, DSM 10512, DSM 10511, DSM 10571, DSM 10576, respectively; or

b) an analogue of the DNA sequence of SEQ ID NO: 1, 7, 9, 11, 13, 15, 21, or 25, respectively, or the DNA sequence obtainable from the plasmid in *Saccharomyces cerevisiae* DSM 9770, DSM 10082, DSM 10080, DSM 10081, *Escherichia coli*, DSM 10512, DSM 10511, DSM 10571, DSM 10576, respectively, which

i) is homologous with the DNA sequence of SEQ ID NO: 1, 7, 9, 11, 13, 15, 21, or 25, respectively, or the DNA sequence obtainable from the plasmid in *Saccharomyces cerevisiae* DSM

9770, DSM 10082, DSM 10080, DSM 10081, *Escherichia coli*, DSM 10512, DSM 10511, DSM 10571, DSM 10576, respectively,

ii) hybridizes with the same oligonucleotide probe as the DNA sequence of SEQ ID NO: 1, 7, 9, 11, 13, 15, 21, or 25 respectively, or the DNA sequence obtainable from the plasmid in *Saccharomyces cerevisiae* DSM 9770, DSM 10082, DSM 10080, DSM 10081, *Escherichia coli*, DSM 10512, DSM 10511, DSM 10571, DSM 10576, respectively,

iii) encodes a polypeptide which is homologous with the polypeptide encoded by a DNA sequence comprising the DNA sequence of SEQ ID NO: 1, 7, 9, 11, 13, 15, 21, or 25 respectively, or the DNA sequence obtainable from the plasmid in *Saccharomyces cerevisiae* DSM 9770, DSM 10082, DSM 10080, DSM 10081, *Escherichia coli*, DSM 10512, DSM 10511, DSM 10571, DSM 10576, respectively,

iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified endoglucanase encoded by the DNA sequence of SEQ ID NO: 1, 7, 9, 11, 13, 15, 21, or 25, respectively, or the DNA sequence obtainable from the plasmid in *Saccharomyces cerevisiae* DSM 9770, DSM 10082, DSM 10080, DSM 10081, *Escherichia coli*, DSM 10512, DSM 10511, DSM 10571, DSM 10576, respectively.

Escherichia coli DSM 10512 was deposited under the Budapest Treaty on 2 February, 1996, at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 16, D-38124 Braunschweig, Germany).

Escherichia coli DSM 10511 was deposited under the Budapest Treaty on 2 February, 1996, at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 16, D-38124 Braunschweig, Germany).

Escherichia coli DSM 10571 was deposited under the Budapest Treaty on 6 March, 1996, at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 16, D-38124 Braunschweig, Germany).

Escherichia coli DSM 10576 was deposited under the Budapest Treaty on 12 March, 1996, at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 16, D-38124 Braunschweig, Germany).

Escherichia coli DSM 10583 was deposited under the Budapest Treaty on 13 March, 1996, at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 16, D-38124 Braunschweig, Germany).

Escherichia coli DSM 10584 was deposited under the Budapest Treaty on 13 March, 1996, at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 16, D-38124 Braunschweig, Germany).

Escherichia coli DSM 10585 was deposited under the Budapest Treaty on 13 March, 1996, at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 16, D-38124 Braunschweig, Germany).

Escherichia coli DSM 10586 was deposited under the Budapest Treaty on 13 March, 1996, at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 16, D-38124 Braunschweig, Germany).

Escherichia coli DSM 10587 was deposited under the Budapest Treaty on 13 March, 1996, at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 16, D-38124 Braunschweig, Germany).

Escherichia coli DSM 10588 was deposited under the Budapest Treaty on 13 March, 1996, at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 16, D-38124 Braunschweig, Germany).

Saccharomyces cerevisiae DSM 9770 was deposited under the Budapest Treaty on 24 February, 1995, at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 16, D-38124 Braunschweig, Germany).

Saccharomyces cerevisiae DSM 10082 was deposited under the Budapest Treaty on 30 June, 1995, at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 16, D-38124 Braunschweig, Germany).

Saccharomyces cerevisiae DSM 10080 was deposited under the Budapest Treaty on 30 June, 1995, at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 16, D-38124 Braunschweig, Germany).

Saccharomyces cerevisiae DSM 10081 was deposited under the Budapest Treaty on 30 June, 1995, at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 16, D-38124 Braunschweig, Germany).

The DNA construct of the invention relating to SEQ ID NO: 1 can be isolated from or produced on the basis of a DNA library of a strain of *Myceliophthora,* in particular a strain of *M. thermophila*, especially *M. thermophila*, CBS 117.65.

The DNA constructs of the invention relating to SEQ ID NOS: 7 and 9 can be isolated from or produced on the basis of a DNA library of a strain of *Acremonium,* especially *Acremonium sp.*, CBS 478.94.

The DNA construct of the invention relating to SEQ ID NO: 11 can be isolated from or produced on the basis of a DNA library of a strain of *Thielavia* in particular a strain of *Thielavia terrestris*, especially *Thielavia terrestris,* NRRL 8126.

The DNA construct of the invention relating to SEQ ID NO: 13 can be isolated from or produced on the basis of a DNA library of a strain of *Macrophomina,* in particular a strain of *M. phaseolina,* especially *M.phaseolina*, CBS 281.96.

The DNA construct of the invention relating to SEQ ID NO: 15 can be isolated from or produced on the basis of a DNA library of a strain of *Crinipellis,* in particular a strain of *C. scabella*, especially *C.scabella*, CBS 280.96.

The DNA construct of the invention relating to SEQ ID NO: 25 can be isolated from or produced on the basis of a DNA library of a strain of *Sordaria,* in particular a strain of *Sordaria fimicola*.

In the present context, the "analogue" of the DNA sequence of SEQ ID NO: 1, 7, 9, 11, 13, 15, 21, or 25, respectively, is intended to indicate any DNA sequence encoding an enzyme exhibiting endoglucanase activity, which has any or all of the properties i)-iv). The analogous DNA sequence

a) may be isolated from another or related (e.g. the same) organism producing the enzyme with endoglucanase activity on the basis of the DNA sequence of SEQ ID NO: 1, 7, 9, 11, 13, 15, 21, or 25, respectively, e.g. using the procedures described herein; the homologue may be an allelic variant of the DNA sequence comprising the DNA sequences shown herein, i.e. an alternative form of a gene that arises through mutation; mutations can be silent (no change in the encoded enzyme) or may encode enzymes having altered amino acid sequence; the homologue of the present DNA sequence may also be a genus or species homologue, i.e. encoding an enzyme with a similar activity derived from another species,

b) may be constructed on the basis of the DNA sequences of SEQ ID NO: 1, 7, 9, 11, 13, 15, 21, or 25, respectively, e.g. by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the endoglucanase encoded by the DNA sequence, but which correspond to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence. However, in the latter case amino acid changes are preferably of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding or activity of the protein, small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain. See in general Ford et al., Protein Expression and Purification 2: 95-107, 1991. Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids

(such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine, valine), aromatic amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine).

It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acids essential to the activity of the polypeptide encoded by the DNA construct of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244, 1081-1085, 1989). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological (i.e. endoglucanase) activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance, crystallography or photoaffinity labeling. See, for example, de Vos et al., Science 255: 306-312, 1992; Smith et al., J. Mol. Biol. 224: 899-904, 1992; Wlodaver et al., FEBS Lett. 309: 59-64, 1992.

The endoglucanase encoded by the DNA sequence of the DNA construct of the invention may comprise a cellulose binding domain (CBD) existing as an integral part of the encoded enzyme, or a CBD from another origin may be introduced into the endoglucanase enzyme thus creating an enzyme hybride. In this context, the term "cellulose-binding domain" is intended to be understood as defined by Peter Tomme et al. "Cellulose-Binding Domains: Classification and Properties" in "Enzymatic Degradation of Insoluble Carbohydrates", John N. Saddler and Michael H. Penner (Eds.), ACS Symposium Series, No. 618, 1996. This definition classifies more than 120 cellulose-binding domains (CBDs) into 10 families (I-X), and it demonstrates that CBDs are found in various enzymes such as cellulases, xylanases, mannanases, arabinofuranosidases, acetyl esterases and chitinases. CBDs have also been found in algae, e.g., the red alga *Porphyra purpurea* as a non-hydrolytic polysaccharide-binding protein, for reference see Peter Tomme et al., supra. However, most of the CBDs are from cellulases and xylanases. CBDs are found at the N or C termini of proteins or are internal. Enzyme hybrids are known in the art, see e.g. WO 90/00609 and WO 95/16782, and may be prepared by transforming into a host cell a DNA construct comprising at least a fragment of DNA encoding the cellulose-binding domain ligated, with or without a linker, to a DNA sequence encoding the enzyme of interest and growing the host cell to express the fused gene. Enzyme hybrids may be described by the following formula:

CBD - MR - X,

wherein CBD is the N-terminal or the C-terminal region of an amino acid sequence corresponding to at least the cellulose-binding domain; MR is the middle region (the linker), and may be a bond, or a short linking group preferably of from about 2 to about 100 carbon atoms, more preferably of from 2 to 40 carbon atoms; or is preferably from about 2 to about 100 amino acids, more preferably of from 2 to 40 amino acids; and X is an N-terminal or C-terminal region of a polypeptide encoded by the DNA sequence of the invention.

The homology referred to in i) above is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., *Journal of Molecular Biology*, 48: 443-453, 1970). Using GAP with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the DNA sequence exhibits a degree of identity preferably of at least 60%, more preferably at least 65%, more preferably at least 70%, even more preferably at least 80%, especially at least 90%, with the coding region of the DNA sequence of SEQ ID NO: 1, 4, 6, 8, 10, 12, or 16, respectively, or the DNA sequence obtainable from the plasmid in *Saccharomyces cerevisiae*, DSM 9770, DSM 10082, DSM 10080, DSM 10081, *Escherichia coli*, DSM 10512, DSM 10511, DSM 10571, or DSM 10576, respectively.

The hybridization referred to in ii) above is intended to indicate that the analogous DNA sequence hybridizes to the same probe as the DNA sequence encoding the endoglucanase enzyme under certain specified conditions which are described in detail in the Materials and Methods section hereinafter. The oligonucleotide probe to be used is the DNA sequence corresponding to the endoglucanase encoding part of the DNA sequence of SEQ ID NO: 1, 7, 9, 11, 13, 15 or 21, respectively, or the DNA sequence obtainable from the plasmid in *Saccharomyces cerevisiae*, DSM 9770, DSM 10082, DSM 10080, DSM 10081, *Escherichia coli*, DSM 10512, DSM 10511, DSM 10571 or DSM 10576, respectively.

The homology referred to in iii) above is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., *Journal of Molecular Biology*, 48: 443-453, 1970). Using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1, the polypeptide encoded by an analogous DNA sequence exhibits a degree of identity preferably of at least 55%, more preferably at least 60%, more preferably at least 65%, even more preferably at least 70%, more preferably at least 80%,

especially at least 90%, with the enzyme encoded by a DNA construct comprising the DNA sequence of SEQ ID NO: 1, 7, 9, 11, 13, 15, 21, or 25, respectively, or the DNA sequence obtainable from the plasmid in *Saccharomyces cerevisiae*, DSM 9770, DSM 10082, DSM 10080, DSM 10081, *Escherichia coli*, DSM 10512, DSM 10511, DSM 10571 or DSM 10576, respectively.

In connection with property iv) above it is intended to indicate an endoglucanase encoded by a DNA sequence isolated from strain *Saccharomyces cerevisiae*, DSM 9770, DSM 10082, DSM 10080, DSM 10081, *Escherichia coli*, DSM 10512, DSM 10511, DSM 10571 or DSM 10576, respectively, and produced in a host organism transformed with said DNA sequence or the corresponding endoglucanase naturally produced by *Myceliophthora thermophila, Acremonium sp., Thielavia terrestris, Macrophomina phaseolina, Crinipellis scabella, Volutella colletotrichoides*, or *Sordaria fimicola*, respectively. The immunological reactivity may be determined by the method described in the Materials and Methods section below.

In further aspects the invention relates to an expression vector harbouring a DNA construct of the invention, a cell comprising the DNA construct or expression vector and a method of producing an enzyme exhibiting endoglucanase activity which method comprises culturing said cell under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

In a still further aspect the invention relates to an enzyme exhibiting endoglucanase activity, which enzyme

a) is encoded by a DNA construct of the invention

b) produced by the method of the invention, and/or

c) is immunologically reactive with an antibody raised against a purified endoglucanase encoded by the DNA sequence of SEQ ID NO: 1, 7, 9, 11, 13, 15, or 21, respectively, or the DNA sequence obtainable from the plasmid in *Saccharomyces cerevisiae*, DSM 9770, DSM 10082, DSM 10080, DSM 10081, *Escherichia coli*, DSM 10512, DSM 10511, DSM 10571 or DSM 10576, respectively.

The endoglucanase mentioned in c) above may be encoded by the DNA sequence isolated from the strain *Saccharomyces cerevisiae*, DSM 9770, DSM 10082, DSM 10080, DSM 10081, *Escherichia coli*, DSM 10512, DSM 10511, DSM 10571 or DSM 10576, respectively, and produced in a host organism transformed with said DNA sequence or the corresponding endoglucanase naturally produced by *Myceliophthora thermophila, Acremonium sp., Thielavia terrestris, Macrophomina phaseolina, Crinipellis scabella, Volutella colletotrichoides* or *Sordaria fimicola*, respectively.

Generally, in the present context the term "enzyme" is understood to include a mature protein or a precursor form thereof as well to a functional fragment thereof which essentially has the activity of the full-length enzyme. Furthermore, the term "enzyme" is intended to include homologues of said enzyme.

Homologues of the present enzyme may have one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding or activity of the protein, small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain. See in general Ford et al., Protein Expression and Purification 2: 95-107, 1991. Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine, valine), aromatic amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine).

It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active enzyme. Amino acids essential to the activity of the enzyme of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham, 1989). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for cellulytic activity to identify amino acid residues that are critical to the activity of the molecule. Sites of ligand-receptor interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance, crystallography or photoaffinity labelling. See, for example, de Vos et al., 1992; Smith et al., 1992, Wlodaver et al., 1992.

The homologue may be an allelic variant, i.e. an alternative form of a gene that arises through mutation, or an altered enzyme encoded by the mutated gene, but having substantially the same activity as the enzyme of the invention. Hence mutations can be silent (no change in the encoded enzyme) or may encode enzymes having altered amino acid sequence.

The homologue of the present enzyme may also be a genus or species homologue, i.e. an enzyme with a similar activity derived from another species.

A homologue of the enzyme may be isolated by using the procedures described herein.

Molecular screening and cloning by polymerase chain reaction (PCR)

Molecular screening for DNA sequences of the invention may be carried out by polymerase chain reaction (PCR) using genomic DNA or double-stranded cDNA isolated from a suitable source, such as any of the herein mentioned organisms, and synthetic oligonucleotide primers prepared on the basis of the DNA sequences or the amino acid sequences disclosed herein. For instance, suitable oligonucleotide primers may be the primers described in the Materials and Methods section.

In accordance with well-known procedures, the PCR fragment generated in the molecular screening may be isolated and subcloned into a suitable vector. The PCR fragment may be used for screening DNA libraries by e.g. colony or plaque hybridization.

Expression cloning in yeast

The DNA sequence of the invention encoding an enzyme exhibiting endoglucanase activity may be isolated by a general method involving

- cloning, in suitable vectors, a DNA library from a suitable source, such as any of the herein mentioned organisms
- transforming suitable yeast host cells with said vectors,
- culturing the host cells under suitable conditions to express any enzyme of interest encoded by a clone in the DNA library,
- screening for positive clones by determining any endoglucanase activity of the enzyme produced by such clones, and
- isolating the enzyme encoding DNA from such clones.

The general method is further disclosed in WO 94/14953 the contents of which are hereby incorporated by reference. A more detailed description of the screening method is given in Example 1 below.

The DNA sequence coding for the enzyme may for instance be isolated by screening a cDNA library of *Macrophomina phaseolina, Crinipellis scabella, Sordaria fimicola* or *Volutella colletotrichoides*, and selecting for clones expressing the appropriate enzyme activity (i.e. endoglucanase activity) or from *Escherichia coli* DSM 10512 deposited under the Budapest Treaty on 2 February, 1996, at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 16, D-38124 Braunschweig, Germany), or from *Escherichia coli* DSM 10511 deposited under the Budapest Treaty on 2 February, 1996, at DSM, or from Escherichia coli DSM 10576, deposited under the Budapest Treaty on 12 March, 1996, at DSM; or from *Escherichia coli* DSM 10571 deposited under the Budapest Treaty on 6 March, 1996, at DSM; or by screening a cDNA library of *Myceliphthora thermophila,* CBS 117.65, *Acremonium sp.*, CBS 478.94, or *Thielavia*

terrestris, NRRL 8126, and selecting for clones expressing the appropriate enzyme activity (i.e. endoglucanase activity) or from *Saccharomyces cerevisiae* DSM 9770 deposited under the Budapest Treaty on 24 February, 1995, at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 16, D-38124 Braunschweig, Germany), or from *Saccharomyces cerevisiae* DSM 10082 deposited under the Budapest Treaty on 30 June, 1995, at DSM, from *Saccharomyces cerevisiae* DSM 10080 deposited under the Budapest Treaty on 30 June, 1995, or from *Saccharomyces cerevisiae* DSM 10081 deposited under the Budapest Treaty on 30 June, 1995, at DSM. The appropriate DNA sequence may then be isolated from the clone by standard procedures, e.g. as described in Example 1.

Nucleic acid construct

As used herein the term "nucleic acid construct" is intended to indicate any nucleic acid molecule of cDNA, genomic DNA, synthetic DNA or RNA origin. The term "construct" is intended to indicate a nucleic acid segment which may be single- or double-stranded, and which may be based on a complete or partial naturally occurring nucleotide sequence encoding an enzyme of interest. The construct may optionally contain other nucleic acid segments.

The nucleic acid construct encoding the enzyme of the invention may suitably be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the enzyme by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., 1989).

The nucleic acid construct encoding the enzyme may also be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by Beaucage and Caruthers, (1981), or the method described by Matthes et al., (1984). According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

Furthermore, the nucleic acid construct may be of mixed synthetic and genomic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire nucleic acid construct, in accordance with standard techniques.

The nucleic acid construct may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202 or Saiki et al., (1988).

The nucleic acid construct is preferably a DNA construct which term will be used exclusively in this specification and claims.

Recombinant vector

A recombinant vector comprising a DNA construct encoding the enzyme of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the DNA sequence encoding the enzyme of the invention is operably linked to additional segments required for transcription of the DNA. In general, the expression vector is derived from plasmid or viral DNA, or may contain elements of both. The term, "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and proceeds through the DNA sequence coding for the enzyme.

The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255 (1980), 12073 - 12080; Alber and Kawasaki, J. Mol. Appl. Gen. 1 (1982), 419 - 434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4,599,311) or ADH2-4c (Russell et al., Nature 304 (1983), 652 - 654) promoters.

Examples of suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. 4 (1985), 2093 - 2099) or the tpiA promoter. Examples of other useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* or *A. awamori* glucoamylase (gluA), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. Preferred are the TAKA-amylase and gluA promoters.

Examples of suitable promoters for use in bacterial host cells include the promoter of the *Bacillus stearothermophilus* maltogenic amylase gene, the *Bacillus licheniformis* alpha-amylase gene, the *Bacillus amyloliquefaciens* BAN amylase gene, the *Bacillus subtilis* alkaline protease gen,

or the *Bacillus pumilus* xylosidase gene, or by the phage Lambda P_R or P_L promoters or the *E. coli* lac, trp or tac promoters.

The DNA sequence encoding the enzyme of the invention may also, if necessary, be operably connected to a suitable terminator.

The recombinant vector of the invention may further comprise a DNA sequence enabling the vector to replicate in the host cell in question.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130). For filamentous fungi, selectable markers include amdS, pyrG, argB, niaD, sC.

To direct an enzyme of the present invention into the secretory pathway of the host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) may be provided in the recombinant vector. The secretory signal sequence is joined to the DNA sequence encoding the enzyme in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the enzyme. The secretory signal sequence may be that normally associated with the enzyme or may be from a gene encoding another secreted protein.

For secretion from yeast cells, the secretory signal sequence may encode any signal peptide which ensures efficient direction of the expressed enzyme into the secretory pathway of the cell. The signal peptide may be naturally occurring signal peptide, or a functional part thereof, or it may be a synthetic peptide. Suitable signal peptides have been found to be the α-factor signal peptide (cf. US 4,870,008), the signal peptide of mouse salivary amylase (cf. O. Hagenbuchle et al., Nature 289, 1981, pp. 643-646), a modified carboxypeptidase signal peptide (cf. L.A. Valls et al., Cell 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), or the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., Yeast 6, 1990, pp. 127-137).

For efficient secretion in yeast, a sequence encoding a leader peptide may also be inserted downstream of the signal sequence and upstream of the DNA sequence encoding the enzyme. The function of the leader peptide is to allow the expressed enzyme to be directed from the endoplasmic reticulum to the Golgi apparatus and further to a secretory vesicle for secretion into the culture medium (i.e. exportation of the enzyme across the cell wall or at least through the cellular membrane into the periplasmic space of the yeast cell). The leader peptide may be the yeast α-factor leader (the use of which is described in e.g. US 4,546,082, EP 16 201, EP 123 294, EP 123 544 and EP 163 529). Alternatively, the leader peptide may be a synthetic leader peptide, which is to say a

leader peptide not found in nature. Synthetic leader peptides may, for instance, be constructed as described in WO 89/02463 or WO 92/11378.

For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease, a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase.

The procedures used to ligate the DNA sequences coding for the present enzyme, the promoter and optionally the terminator and/or secretory signal sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., op.cit.).

Host cells

The DNA sequence encoding the present enzyme introduced into the host cell may be either homologous or heterologous to the host in question. If homologous to the host cell, i.e. produced by the host cell in nature, it will typically be operably connected to another promoter sequence or, if applicable, another secretory signal sequence and/or terminator sequence than in its natural environment. The term "homologous" is intended to include a cDNA sequence encoding an enzyme native to the host organism in question. The term "heterologous" is intended to include a DNA sequence not expressed by the host cell in nature. Thus, the DNA sequence may be from another organism, or it may be a synthetic sequence.

The host cell into which the DNA construct or the recombinant vector of the invention is introduced may be any cell which is capable of producing the present enzyme and includes bacteria, yeast, fungi and higher eukaryotic cells.

Examples of bacterial host cells which, on cultivation, are capable of producing the enzyme of the invention are gram-positive bacteria such as strains of *Bacillus*, such as strains of *B. subtilis, B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus, B. megatherium* or *B. thuringiensis*, or strains of *Streptomyces*, such as *S. lividans* or *S. murinus*, or gram-negative bacteria such as *Echerichia coli*. The transformation of the bacteria may be effected by protoplast transformation or by using competent cells in a manner known per se (cf. Sambrook et al., supra).

When expressing the enzyme in bacteria such as *E. coli*, the enzyme may be retained in the cytoplasm, typically as insoluble granules (known as inclusion bodies), or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed and the

granules are recovered and denatured after which the enzyme is refolded by diluting the denaturing agent. In the latter case, the enzyme may be recovered from the periplasmic space by disrupting the cells, e.g. by sonication or osmotic shock, to release the contents of the periplasmic space and recovering the enzyme.

Examples of suitable yeasts cells include cells of *Saccharomyces* spp. or *Schizosaccharomyces* spp., in particular strains of *Saccharomyces cerevisiae* or *Saccharomyces kluyveri*. Methods for transforming yeast cells with heterologous DNA and producing heterologous enzymes therefrom are described, e.g. in US 4,599,311, US 4,931,373, US 4,870,008, 5,037,743, and US 4,845,075, all of which are hereby incorporated by reference. Transformed cells are selected by a phenotype determined by a selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient, e.g. leucine. A preferred vector for use in yeast is the POT1 vector disclosed in US 4,931,373. The DNA sequence encoding the enzyme of the invention may be preceded by a signal sequence and optionally a leader sequence, e.g. as described above. Further examples of suitable yeast cells are strains of *Kluyveromyces*, such as *K. lactis*, *Hansenula*, e.g. *H. polymorpha*, or *Pichia*, e.g. *P. pastoris* (cf. Gleeson et al., J. Gen. Microbiol. 132, 1986, pp. 3459-3465; US 4,882,279).

Examples of other fungal cells are cells of filamentous fungi, e.g. *Aspergillus* spp., *Neurospora* spp., *Fusarium* spp. or *Trichoderma* spp., in particular strains of *A. oryzae*, *A. nidulans*, *A. niger*, or *Fusarium graminearum*. The use of *Aspergillus* spp. for the expression of proteins is described in, e.g., EP 272 277, EP 230 023. The transformation of *F. oxysporum* may, for instance, be carried out as described by Malardier et al., 1989, Gene 78: 147-156.

When a filamentous fungus is used as the host cell, it may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome to obtain a recombinant host cell. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination.

The transformed or transfected host cell described above is then cultured in a suitable nutrient medium under conditions permitting the expression of the present enzyme, after which the resulting enzyme is recovered from the culture.

The medium used to culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection). The enzyme produced by the cells

may then be recovered from the culture medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like, dependent on the type of enzyme in question.

In a still further aspect, the present invention relates to a method of producing an enzyme according to the invention, wherein a suitable host cell transformed with a DNA sequence encoding the enzyme is cultured under conditions permitting the production of the enzyme, and the resulting enzyme is recovered from the culture.

Enzyme Screening driven by taxonomy as well as ecology:

A powerful tool like the molecular screening disclosed herein, designed to detect and select said type of interesting enzymes, can still not stand on its own. In order to maximize the chances of making interesting discoveries the molecular screening approach was in the present investigation combined with careful selection of which fungi to screen. The selection was done through a thorough insight in the identification of fungi, in taxonomical classification and in phylogenetic relationships.

A taxonomic hot spot for production of cellulytic enzymes can further only be fully explored if also the ecological approach is included. Thorough knowledge about the adaptation to various substrates (especially saprotrophic, necrotrophic or biotrophic degradation of plant materials) are prerequisites for designing an intelligent screening and for managing a successful selection of strains and ecological niches to be searched.

Both the taxonomy and the ecological approach disclosed herein aim at maximizing discovery of said enzymes in the molecular screening program. However, still several hundreds (or if all preliminary work is included) several thousand fungi have been brought in culture in order to detect the 53 hits of said type of cellulytic enzyme here reported.

The screening and cloning may be carried out using the following:

MATERIALS AND METHODS

List of organisms:

Saccharomyces cerevisiae, DSM 9770, DSM 10082, DSM 10080, DSM 10081, or *Escherichia coli*, DSM 10512, DSM 10511, DSM 10571, DSM 10576, respectively, containing the plasmid comprising the full length DNA sequence, coding for the endoglucanase of the invention, in the shuttle vector pYES 2.0.

Escherichia coli DSM 10583, 10584, 10585, 10586, 10587, and 10588.

Diplodia gossypina Cooke

Deposit of Strain, Acc No: CBS 274.96

Classification: Ascomycota, Loculoascomycetes, Dothideales, Cucurbitariaceae

Ulospora bilgramii (Hawksw. et al.) Hawksw. et al.

Acc No of strain: NKBC 1444, Nippon University, (Prof. Tubaki collection)

Classification: Ascomycota, Loculoascomycetes, Dothideales, (family unclassified)

Microsphaeropsis sp.

Isolated from: Leaf of Camellia japonica (Theaceae, Guttiferales), grown in Kunming Botanical garden, Yunnan Province, China

Classification: Ascomycota, Loculoascomycetes, Dothideales, (family unclassified)

Macrophomina phaseolina (Tassi) Goidannich

Syn: Rhizoctonia bataticola

Deposit of Strain, Acc No.:CBS 281.96

Isolated from seed of Glycine max (Leguminosa), cv CMM 60, grown in Thailand, 1990

Classification: Ascomycota, Discomycetes, Rhytismatales, Rhytismataceae

Ascobolus stictoideus Speg.

Isolated from goose dung, Svalbard, Norway

Classification: Ascomycota, Discomycetes, Pezizales, Ascobolaceae

Saccobolus dilutellus (Fuck.) Sacc.

Deposit of strain: Acc No CBS 275.96

Classification: Ascomycota, Discomycetes, Pezizales, Ascobolaceae

Penicillium verruculosum Peyronel

Ex on Acc No of species: ATCC 62396

Classification: Ascomycota, Plectomycetes, Eurotiales, Trichocomaceae

Penicillium chrysogenum Thom

Acc No of Strain: ATCC 9480

Classification: Ascomycota, Plectomycetes, Eurotiales, Trichocomaceae

Thermomyces verrucosus Pugh et al

Deposit of Strain, Acc No.: CBS 285.96

Classification: Ascomycota, Plectomycetes, Eurotiales, (family unclassified; affiliation based on 18S RNA, sequencing and homologies)

Xylaria hypoxylon L. ex Greville

Deposit of Strain, Acc No: CBS 284.96

Classification: Ascomycota, Pyrenomycetes, Xylariales, Xylariaceae

Poronia punctata (Fr.ex L.) Fr.

Classification: Ascomycota, Pyrenomycetes, Xylariales, Xylariaceae

Nodulisporum sp

Isolated from leaf of Camellia reticulatá (Theaceae, Guttiferales), grown in Kunming Botanical Garden, Yunnan Province, China

Classification: Ascomycota, Pyrenomycetes, Xylariales, Xylariaceae

Cylindrocarpon sp

Isolated from marine sample, the Bahamas

Classification: Ascomycota, Pyrenomycetes, Hypocreales (unclassified)

Acremonium sp

Deposit of Strain, Acc. No.: CBS 478.94

Classification: Ascomycota, Pyrenomycetes, Hypocreales, Hypocreaceae

Fusarium anguioides Sherbakoff

Acc No of strain: IFO 4467

Classification: Ascomycota, Pyrenomycetes, Hypocreales, Hypocreaceae

Fusarium poae (Peck) Wr.

Ex on Acc No of species: ATCC 60883

Classification: Ascomycota, Pyrenomycetes, Hypocreales, Hypocreaceae

Fusarium solani (Mart.)Sacc.emnd.Snyd & Hans.

Acc No of strain: IMI 107.511

Classification: Ascomycota, Pyrenomycetes, Hypocreales, Hypocreaceae

Fusarium oxysporum ssp lycopersici (Sacc.)Snyd. & Hans.

Acc No of strain: CBS 645.78

Classification: Ascomycota, Pyrenomycetes, Hypocreales, Hypocreaceae

Fusarium oxysporum ssp passiflora

Acc No of strain: CBS 744.79

Classification: Ascomycota, Pyrenomycetes, Hypocreales, Hypocreaceae

Gliocladium catenulatum Gillman & Abbott

Acc. No. of strain: CBS 227.48

Classification: Ascomycota, Pyrenomycetes, Hypocreales, Hypocreaceae

Nectria pinea Dingley

Deposit of Strain, Acc. No. CBS 279.96

Classification: Ascomycota, Pyrenomycetes, Hypocreales, Nectriaceae

Volutella colletotrichoides

Acc No of Strain: CBS 400.58

Classification: Ascomycota, Pyrenomycetes, Hypocreales (unclassified)

Sordaria macrospora Auerswald

Ex on Acc No of species: ATCC 60255

Classification: Ascomycota, Pyrenomycetes, Sordariales, Sordariaceae

Sordaria fimicola (Roberge) Cesati et De Notaris

Ex on Acc. No. for the species: ATCC 52644

Isolated from dung by H.Dissing, ISP, KU, Denmark

Classification: Ascomycota, Pyrenomycetes, Sordariales, Sordariaceae

Humicola grisea Traeen

ex on Acc No for the species: ATCC 22726

Source: Hatfield Polytechnic

Classification: Ascomycota, Pyrenomycetes, Sordariales, (fam. unclassified)

Humicola nigrescens Omvik

Acc No of strain: CBS 819.73

Classification: Ascomycota, Pyrenomycetes, Sordariales, (fam. unclassified)

Scytalidium thermophilum (Cooney et Emerson) Austwick

Acc No of strain: ATCC 28085

Classification: Ascomycota, Pyrenomycetes, Sordariales, (fam. unclassified)

Thielavia thermophila Fergus et Sinden

(syn Corynascus thermophilus)

Acc No of strain: CBS 174.70, IMI 145.136

Classification: Ascomycota, Pyrenomycetes, Sordariales, Chaetomiaceae

Isolated from Mushroom compost

Thielavia terrestris (Appinis) Malloch et Cain

Acc No of strain: NRRL8126

Classification: Ascomycota, Pyrenomycetes, Sordariales, Chaetomiaceae

Cladorrhinum foecundissimum Saccardo et Marchal

Ex on Acc No of species: ATCC 62373

Classification: Ascomycota, Pyrenomycetes, Sordariales, Lasiosphaeriaceae

Isolated from leaf of Selandin sp. (Compositaceae, Asterales), Dallas Mountain, Jamaica

Syspastospora boninensis

Acc No of strain: NKBC 1515 (Nippon University, profe Tubaki Collection)

Classification: Ascomycota, Pyrenomycetes, Sordariales, Cerastomataceae

Chaetomium cuniculorum Fuckel

Acc. No. of strain: CBS 799.83

Classification: Ascomycota, Pyrenomycetes, Sordariales, Chaetomiaceae

Chaetomium brasiliense Batista et Potual

Acc No of strain: CBS 122.65

Classification: Ascomycota, Pyrenomycetes, Sordariales, Chaetomiaceae

Chaetomium murorum Corda

Acc No of strain: CBS 163.52

Classification: Ascomycota, Pyrenomycetes, Sordariales, Chaetomiaceae

Chaetomium virescens (von Arx) Udagawa

Acc.No. of strain: CBS 547.75

Classification: Ascomycota, Pyrenomycetes, Sordariales, Chaetomiaceae

Myceliophthora thermophila (Apinis) Oorschot

Deposit of Strain, Acc No:CBS 117.65

Classification: Ascomycota, Pyrenomycetes, Sordariales, Chaetomiaceae

Nigrospora sp

Deposit of strain, Acc No: CBS 272.96

Isolated from leaf of Artocarpus altilis, Moraceae, Urticales grown in Christiana, Jamaica

Classification: Ascomycota, Pyrenomycetes, Trichosphaeriales, (family unclassified)

Nigrospora sp

Isolated from leaf of Pinus yuannanensis, Botanical Garden, Kuning, Yunnan.

Classification: Ascomycota, Pyrenomycetes, Trichosphaeriales, Abietaceae, Pinales.

Diaporthe syngenesia

Deposit of strain, Acc No: CBS 278.96

Classification: Ascomycota, Pyrenomycetes, Diaporthales, Valsaceae

Colletotrichum lagenarium (Passerini) Ellis et Halsted

syn Glomerella cingulata var orbiculare Jenkins et Winstead

Ex on acc No of species: ATCC 52609

Classification: Ascomycota, Pyrenomycetes, Phyllachorales

Exidia glandulosa Fr.

Deposit of Strain, Acc No: CBS 277.96

Classification: Basidiomycota, Hymenomycetes, Auriculariales, Exidiaceae

Crinipellis scabella (Alb.&Schw.:Fr.)Murr

Deposit of strain: Acc No CBS 280.96

Classification: Basidiomycota, Hymenomycetes, Agaricales,

Panaeolus retirugis (Fr.) Gill.

Acc.No. of strain: CBS 275.47

Classification: Basidiomycota, Hymenomycetes, Agaricales, Coprinaceae

Fomes fomentarius (L.) Fr.

Deposit of strain: Acc No. CBS 276.96

Classification: Basidiomycota, Hymenomycetes, Aphyllophorales, Fomitaceae

Spongipellis sp.

Deposit of Strain: Acc No CBS 283.96

Classification: Basidiomycota, Hymenomycetes, Aphyllophorales,

Bjerkanderaceae (identified and affiliated taxonomically by 18S sequence and homology)

Trametes sanguinea (Fr.) Lloyd

syn: Polyporus sanguineus; Pycnoporus sanguineus (L.:Fr.) Murrill

Acc No of strain: AKU 5062 (Kyoto University Culture Collection)

Classification: Basidiomycota, Aphyllophorales, Polyporaceae

Schizophyllum commune Fr

Acc. No. of species: ATCC 38548

Classification: Basidiomycota, Aphyllophorales, Schizophyllaceae

Rhizophlyctis rosea (de Bary & Wor) Fischer

Deposit of Strain: Acc No.: CBS 282.96

Classification: Chytridiomycota, Chytridiomycetes, Spizellomycetales, Spizellomycetaceae

Rhizomucor pusillus (Lindt) Schipper

syn: Mucor pusillus

Acc No of strain: IFO 4578

Ex on Acc No of species: ATCC 46883

Classification: Zygomycota, Zygomycetes, Mucorales, Mucoraceae

Phycomyces nitens (Kunze) van Tieghem & Le Monnier

Acc No of strain: IFO 4814

Ex on Acc No of species: ATCC 16327

Classification: Zygomycota, Zygomycetes, Mucorales, Mucoraceae

Chaetostylum fresenii van Tieghem & Le Monnier

syn. Helicostylum fresenii

Acc No of strain NRRL 2305

Classification: Zygomycota, Zygomycetes, Mucorales, Thamnidiaceae

Unclassified:

Trichothecium roseum

Acc No of strain: IFO 5372

Coniothecium sp

Endophyte, isolated from leaf of unidentifed higher plant, growing in Kunming, Yunnan, China

Unclassified and Un-identified:

Deposit of strain, Acc No.: CBS 271.96

Isolated from leaf of Artocarpus altilis (Moraceae, Urticales), grown in Christiana, Jamaica

Deposit of strain, Acc No.: CBS 273.96

Isolated from leaf of Pimenta dioica (Myrtaceae, Myrtales) grown in Dallas Mountain, Jamaica

Deposit of strain: CBS 270.96

Isolated from leaf of Pseudocalymma alliaceum (Bignoniaceae, Solanales) growing in Dallas Mountain, Jamaica

Other strains:

Escherichia coli MC1061 and DH10B.

Yeast strain: The *Saccharomyces cerevisiae* strain used was W3124 (MATα; ura 3-52; leu 2-3, 112; his 3-D200; pep 4-1137; prc1::HIS3; prb1:: LEU2; cir+).

Plasmids:

The *Aspergillus* expression vector pHD414 is a derivative of the plasmid p775 (described in EP 238 023). The construction of pHD414 is further described in WO 93/11249.

pYES 2.0 (Invitrogen)

pA2C477, pA2C193, pA2C357, pA2C371, pA2C385, pA2C475, pA2C488, pA2C502 (See example 1, 2, 3 and 4).

Isolation of the DNA sequence of SEQ ID NO: 1, 7, 9, 11, 13, 15, 21, or 25, respectively:

The full length DNA sequence, comprising the cDNA sequence of SEQ ID NO: 1, 7, 9, 11, 13, 15, 21, or 25, respectively, coding for the endoglucanase of the invention, can be obtained from the deposited organism *S. cerevisiae*, DSM 9770, DSM 10082, DSM 10080, DSM 10081, *E. coli*, DSM 10512, DSM 10511, DSM 10571 or DSM 10576, respectively, by extraction of plasmid DNA by

methods known in the art (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY).

PCR primers for molecular screening of cellulases of the present invention:

The four degenerate, deoxyinosine-containing oligonucleotide primers (sense; s and antisense; as1, as2 and as3) corresponding to four highly conserved amino acid regions found in the deduced amino acid sequences of *Thielavia terrestris* cellulase, *Myceliophthora thermophilum* cellulase, and two cellulases from *Acremonium sp.* The residues are numbered according to the *Myceliophthora thermophilum* sequence. The deoxyinosines are depicted by an I in the primer sequences, and the restriction sites are underlined.

```
                          27                                  35
                NH2-      Thr Arg Tyr Trp Asp Cys Cys Lys Pro/Thr -COOH (SEQ ID NO: 79)
s 5'- CCCCAAGCTT          ACI AGI TAC TGG GAC TGC TGC AAA AC -3' (SEQ ID NO: 84)
      HindIII                 C     T       T   T   T   G C

                          106                 111
                NH2-      Trp Cys Cys Ala Cys Tyr -COOH (SEQ ID NO: 81)
      as1 3'-              CC ACA ACA CGI ACA AT    AGATCTGATC -5' (SEQ ID NO: 85)
                              G   G       G          XbaI

                          145                                 152
                NH2-      Pro Gly Gly Gly Leu/Val Gly Ile/Leu Phe -COOH (SEQ ID NO: 82)
      as2 3'-             GGI CCI CCI CCI AAI     CCI AAI     AA   AGATCTGATC -5' (SEQ
                                      C           G              XbaI   ID NO: 86)
                                      G           T

                          193                             198
                NH2-      Trp Arg Phe/Tyr Asp Trp Phe -COOH (SEQ ID NO: 83)
      as3 3'-              CC GCI AAA     CTA ACC AAA  AGATCTGATC -5' (SEQ ID NO: 87)
                              T    TG       G       G   XbaI
```

Molecular screening by polymerase chain reaction (PCR):

In vitro amplification of genomic DNA and double-stranded cDNA.

Directional, double-stranded cDNA was synthesized from 5 µg of poly(A)$^+$ RNA as described below. Genomic DNA was isolated according to Yelton et al.

Approximately 10 to 20 ng of double-stranded, cellulase-induced cDNA or 100 to 200 ng of genomic DNA from a selection of fungal strains was PCR amplified in PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % (w/v) gelatin) containing 200 µM of each dNTP and 100 pmol of each degenerate primer in three combinations:

1) sense,

5'-CCCCAAGCTTACI$^{A}/_{C}$GITA$^{C}/_{T}$TGGGA$^{C}/_{T}$TG$^{C}/_{T}$TG$^{C}/_{T}$AA$^{A}/_{G}$$^{A}/_{C}$C-3' (SEQ ID NO: 84)

antisense 1,

5'- CTAGTCTAGATA$^{A}/_{G}$CAIGC$^{A}/_{G}$CA$^{A}/_{G}$CACC -3' (SEQ ID NO: 85); or

2) sense,

5'- CCCCAAGCTTACI$^{A}/_{C}$GITA$^{C}/_{T}$TGGGA$^{C}/_{T}$TG$^{C}/_{T}$TG$^{C}/_{T}$AA$^{A}/_{G}$$^{A}/_{C}$C-3' (SEQ ID NO: 84)

antisense 2,

CTAGTCTAGAAAIA$^{A}/_{G}/^{T}$ICCIA$^{A}/^{C}/^{G}$ICCICCICCIGG -3' (SEQ ID NO: 86); or

3) sense,

5'- CCCCAAGCTTACI$^{A}/_{C}$GITA$^{C}/_{T}$TGGGA$^{C}/_{T}$TG$^{C}/_{T}$TG$^{C}/_{T}$AA$^{A}/_{G}$$^{A}/_{C}$C-3' (SEQ ID NO: 84)

antisense 3,

5'- CTAGTCTAGAIAACCA$^{A}/_{G}$TCA$^{A}/_{G}$$^{A}/_{T}AIC^{G}/_{T}$CC -3 (SEQ ID NO: 87);

a DNA thermal cycler (Landgraf, Germany) and 2.5 units of Taq polymerase (Perkin-Elmer, Cetus, USA). Thirty cycles of PCR were performed using a cycle profile of denaturation at 94°C for 1 min, annealing at 64°C for 2 min, and extension at 72°C for 3 min. Ten-µl aliquots of the amplification products were analyzed by electrophoresis in 3 % agarose gels (NuSieve, FMC) with HaeIII-digested ϕX174 RF DNA as a size marker.

Direct sequencing of the PCR products.

Eighty-µl aliquots of the PCR products were purified using the QIAquick PCR purification kit (Qiagen, USA) according to the manufacturer's instructions. The nucleotide sequences of the amplified PCR fragments were determined directly on the purified PCR products by the dideoxy chain-termination method, using 50-150 ng template, the Taq deoxy-terminal cycle sequencing kit (Perkin-Elmer, USA), fluorescent labeled terminators and 5 pmol of the sense primer: 5'-CCCCAAGCTTACI$^{A}/_{C}$GITA$^{C}/_{T}$TGGGA$^{C}/_{T}$T-G$^{C}/_{T}$TG$^{C}/_{T}$AA$^{A}/_{G}$$^{A}/_{C}$C-3' (SEQ ID NO: 84). Analysis of the sequence data was performed according to Devereux *et al.*

Cloning by polymerase chain reaction (PCR):

Subcloning of PCR fragments.

Twenty five µl aliquots of the PCR products generated as described above were electrophoresed in 0.8 % low gelling temperature agarose (SeaPlaque GTG, FMC) gels, the relevant fragments were excised from the gels, and recovered by agarase treatment by adding 0.1 vol of 10 x agarase buffer (New England Biolabs) and 2 units per 100 µl molten agarose to the sample, followed by incubation at 45°C for 1.5 h. The sample was phenol and chloroform extracted, and precipitated by addition of 2 vols of 96 % EtOH and 0.1 of 3 M NaAc, pH 5.2. The PCR fragments were recovered by centrifugation, washed in 70 % EtOH, dried and resuspended in 20 µl

of restriction enzyme buffer (10 mM Tris-HCl, 10 mM MgCl2, 50 mM NaCl, 1 mM DTT). The fragments were digested with *Hin*dIII and *Xba*I, phenol and chloroform extracted, recovered by precipitation with 2 vols of 96 % EtOH and 0.1 of 3 M NaAc, pH 5.2, and subcloned into *Hin*dIII/*Xba*I-cleaved pYES 2.0 vector.

Screening of cDNA libraries and characterization of the positive clones

cDNA libraries in S. cerevisiae or *E. coli*, constructed as described below, were screened by colony hybridization (Sambrook, 1989) using the corresponding random-primed (Feinberg and Vogelstein) ^{32}P-labeled (>1 x 10^9 cpm/µg) PCR products as probes. The hybridizations were carried out in 2 x SSC (Sambrook, 1989), 5 x Denhardt's solution (Sambrook, 1989), 0.5 % (w/v) SDS, 100 µg/ml denatured salmon sperm DNA for 20 h at 65°C followed by washes in 5 x SSC at 25°C (2 x 15 min), 2 x SSC, 0.5 % SDS at 65°C (30 min), 0.2 x SSC, 0.5 % SDS at 65°C (30 min) and finally in 5 x SSC (2 x 15 min) at 25°C. The positive cDNA clones were characterized by sequencing the ends of the cDNA inserts with pYES 2.0 polylinker primers (Invitrogen, USA), and by determining the nucleotide seuence of the longest cDNA from both strands by the dideoxy chain termination method (Sanger et al.) using fluorescent labeled terminators. Qiagen purified plasmid DNA (Qiagen, USA) was sequenced with the Taq deoxy terminal cycle sequencing kit (Perkin Elmer, USA) and either pYES 2.0 polylinker primers (Invitrogen, USA) or synthetic oligonucleotide primers using an Applied Biosystems 373A automated sequencer according to the manufacturers instructions. Analysis of the sequence data was performed according to Devereux *et al.*

Extraction of total RNA was performed with guanidinium thiocyanate followed by ultracentrifugation through a 5.7 M CsCl cushion, and isolation of poly(A)$^+$RNA was carried out by oligo(dT)-cellulose affinity chromatography using the procedures described in WO 94/14953.

cDNA synthesis

Double-stranded cDNA was synthesized from 5 μg poly(A)$^+$ RNA by the RNase H method (Gubler and Hoffman (1983) Gene **25**:263-269, Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY) using the hair-pin modification developed by F. S. Hagen (pers. comm.). The poly(A)$^+$ RNA (5 μg in 5 μl of DEPC-treated water) was heated at 70□C for 8 min. in a pre-siliconized, RNase-free Eppendorph tube, quenched on ice and combined in a final volume of 50 μl with reverse transcriptase buffer (50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, Bethesda Research Laboratories) containing 1 mM of dATP, dGTP and dTTP and 0.5 mM 5-methyl-dCTP (Pharmacia), 40 units human placental ribonuclease inhibitor (RNasin, Promega), 1.45 μg of oligo$(dT)_{18}$-Not I primer (Pharmacia) and 1000 units

SuperScript II RNase H reverse transcriptase (Bethesda Research Laboratories). First-strand cDNA was synthesized by incubating the reaction mixture at 45□C for 1 hour. After synthesis, the mRNA:cDNA hybrid mixture was gelfiltrated through a MicroSpin S-400 HR (Pharmacia) spin column according to the manufacturer's instructions.

After the gelfiltration, the hybrids were diluted in 250 μl second strand buffer (20 mM Tris-Cl, pH 7.4, 90 mM KCl, 4.6 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.16 mM βNAD+) containing 200 μM of each dNTP, 60 units *E. coli* DNA polymerase I (Pharmacia), 5.25 units RNase H (Promega) and 15 units *E. coli* DNA ligase (Boehringer Mannheim). Second strand cDNA synthesis was performed by incubating the reaction tube at 16°C for 2 hours and additional 15 min. at 25°C. The reaction was stopped by addition of EDTA to a final concentration of 20 mM followed by phenol and chloroform extractions.

Mung bean nuclease treatment

The double-stranded cDNA was precipitated at -20°C for 12 hours by addition of 2 vols 96% EtOH, 0.2 vol 10 M NH_4Ac, recovered by centrifugation, washed in 70% EtOH, dried and resuspended in 30 μl Mung bean nuclease buffer (30 mM NaAc, pH 4.6, 300 mM NaCl, 1 mM $ZnSO_4$, 0.35 mM DTT, 2% glycerol) containing 25 units Mung bean nuclease (Pharmacia). The single-stranded hair-pin DNA was clipped by incubating the reaction at 30°C for 30 min., followed by addition of 70 μl 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, phenol extraction and precipitation with 2 vols of 96% EtOH and 0.1 vol 3 M NaAc, pH 5.2 on ice for 30 min.

Blunt-ending with T4 DNA polymerase

The double-stranded cDNAs were recovered by centrifugation and blunt-ended in 30 μl T4 DNA polymerase buffer (20 mM Tris-acetate, pH 7.9, 10 mM MgAc, 50 mM KAc, 1 mM DTT) containing 0.5 mM of each dNTP and 5 units T4 DNA polymerase (New England Biolabs) by incubating the reaction mixture at 16°C for 1 hour. The reaction was stopped by addition of EDTA to a final concentration of 20 mM, followed by phenol and chloroform extractions, and precipitation for 12 hours at -20°C by adding 2 vols 96% EtOH and 0.1 vol 3 M NaAc pH 5.2.

Adaptor ligation, Not I digestion and size selection:

After the fill-in reaction the cDNAs were recovered by centrifugation, washed in 70% EtOH and dried. The cDNA pellet was resuspended in 25 μl ligation buffer (30 mM Tris-Cl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP) containing 2.5 μg non-palindromic BstXI adaptors (Invitrogen) and 30 units T4 ligase (Promega) and incubated at 16°C for 12 hours. The reaction was stopped by

heating at 65°C for 20 min. and then cooling on ice for 5 min. The adapted cDNA was digested with Not I restriction enzyme by addition of 20 μl water, 5 μl 10x Not I restriction enzyme buffer (New England Biolabs) and 50 units Not I (New England Biolabs), followed by incubation for 2.5 hours at 37°C. The reaction was stopped by heating at 65°C for 10 min. The cDNAs were size-fractionated by gel electrophoresis on a 0.8% SeaPlaque GTG low melting temperature agarose gel (FMC) in 1x TBE to separate unligated adaptors and small cDNAs. The cDNA was size-selected with a cut-off at 0.7 kb and rescued from the gel by use of β-Agarase (New England Biolabs) according to the manufacturer's instructions and precipitated for 12 hours at -20°C by adding 2 vols 96% EtOH and 0.1 vol 3 M NaAc pH 5.2.

Construction of libraries

The directional, size-selected cDNA was recovered by centrifugation, washed in 70% EtOH, dried and resuspended in 30 μl 10 mM Tris-Cl, pH 7.5, 1 mM EDTA. The cDNAs were desalted by gelfiltration through a MicroSpin S-300 HR (Pharmacia) spin column according to the manufacturer's instructions. Three test ligations were carried out in 10 μl ligation buffer (30 mM Tris-Cl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP) containing 5 μl double-stranded cDNA (reaction tubes #1 and #2), 15 units T4 ligase (Promega) and 30 ng (tube #1), 40 ng (tube #2) and 40 ng (tube #3, the vector background control) of BstXI-NotI cleaved pYES 2.0 vector. The ligation reactions were performed by incubation at 16□C for 12 hours, heating at 70°C for 20 min. and addition of 10 μl water to each tube. 1 μl of each ligation mixture was electroporated into 40 μl electrocompetent *E. coli* DH10B cells (Bethesda research Laboratories) as described (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY). Using the optimal conditions a library was established in *E. coli* consisting of pools. Each pool was made by spreading transformed *E. coli* on LB+ampicillin agar plates giving 15.000-30.000 colonies/plate after incubation at 37°C for 24 hours. 20 ml LB+ampicillin was added to the plate and the cells were suspended herein. The cell suspension was shaked in a 50 ml tube for 1 hour at 37°C. Plasmid DNA was isolated from the cells according to the manufacturer's instructions using QIAGEN plasmid kit and stored at -20°C.

1 μl aliquots of purified plasmid DNA (100 ng/μl) from individual pools were transformed into *S. cerevisiae* W3124 by electroporation (Becker and Guarante (1991) Methods Enzymol. **194**:182-187) and the transformants were plated on SC agar containing 2% glucose and incubated at 30°C.

Identification of positive colonies

After 3-5 days of growth, the agar plates were replica plated onto a set of SC + galactose-uracil agar plates containing 0.1% AZCL HE cellulose. These plates were incubated for 3-7 days at 30□C. Endoglucanase positive colonies were identified as colonies surrounded by a blue halo.

Cells from enzyme-positive colonies were spread for single colony isolation on agar, and an enzyme-producing single colony was selected for each of the endoglucanase-producing colonies identified.

Characterization of positive clones

The positive clones were obtained as single colonies, the cDNA inserts were amplified directly from the yeast colony using biotinylated polylinker primers, purified by magnetic beads (Dynabead M-280, Dynal) system and characterized individually by sequencing the 5'-end of each cDNA clone using the chain-termination method (Sanger et al. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**:5463-5467) and the Sequenase system (United States Biochemical).

The nucleotide sequence was determined of the longest cDNA from both strands by the dideoxy chain termination method (Sanger et al.) using fluorescent labeled terminators. Plasmid DNA was rescued by transformation into *E. coli* as described below. Qiagen purified plasmid DNA (Qiagen, USA) was sequenced with the Taq deoxy terminal cycle sequencing kit (Perkin Elmer, USA) and either pYES 2.0 polylinker primers (Invitrogen, USA) or synthetic oligonucleotide primers using an Applied Biosystems 373A automated sequencer according to the manufacturers instructions. Analysis of the sequence data was performed according to Devereux *et al.*

Isolation of a cDNA gene for expression in *Aspergillus*

An endoglucanase-producing yeast colony was inoculated into 20 ml YPD broth in a 50 ml glass test tube. The tube was shaken for 2 days at 30°C. The cells were harvested by centrifugation for 10 min. at 3000 rpm.

DNA was isolated according to WO 94/14953 and dissolved in 50 μl water. The DNA was transformed into *E. coli* by standard procedures. Plasmid DNA was isolated from *E. coli* using standard procedures, and analyzed by restriction enzyme analysis. The cDNA insert was excised using appropriate restriction enzymes and ligated into an *Aspergillus* expression vector.

Transformation of *Aspergillus oryzae* or *Aspergillus niger*

Protoplasts may be prepared as described in WO 95/02043, p. 16, line 21 - page 17, line 12, which is hereby incorporated by reference.

100 µl of protoplast suspension is mixed with 5-25 µg of the appropriate DNA in 10 µl of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5, 10 mM $CaCl_2$). Protoplasts are mixed with p3SR2 (an *A. nidulans* amdS gene carrying plasmid). The mixture is left at room temperature for 25 minutes. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution is added and carefully mixed. The mixture is left at room temperature for 25 minutes, spun at 2500 g for 15 minutes and the pellet is resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are spread on minimal plates (Cove, Biochem. Biophys. Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked and spread for single colonies. This procedure is repeated and spores of a single colony after the second reisolation is stored as a defined transformant.

Test of *A. oryzae* transformants

Each of the transformants were inoculated in 10 ml YPM and propagated. After 2-5 days of incubation at 37°C, 10 ml supernatant was removed. The endoglucanase activity was identified by AZCL HE cellulose as described above.

Hybridization conditions (to be used in evaluating property ii) of the DNA construct of the invention): Suitable conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (standard saline citrate) for 10 min, and prehybridization of the filter in a solution of 5 x SSC (Sambrook et al. 1989), 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* **132**:6-13), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at ca. 45°C. The filter is then washed two times for 30 minutes in 2 x SSC, 0.5 % SDS at preferably not higher than 50°C, more preferably not higher than 55°C, more preferably not higher than 60°C, more preferably not higher than 65°C, even more preferably not higher than 70°C, especially not higher than 75°C.

The nucleotide probe to be used in the hybridization is the DNA sequence corresponding to the endoglucanase encoding part of the DNA sequence of SEQ ID NO: 1, 7, 9, 11, 13, 15, 21, or 25, resepctively, and/or the DNA sequence obtainable from the plasmid in *S. cerevisiae*, DSM 9770,

DSM 10082, DSM 10080, DSM 10081, *E. coli*, DSM 10512, DSM 10511, DSM 10571 or DSM 10576, respectively.

Immunological cross-reactivity

Antibodies to be used in determining immunological cross-reactivity may be prepared by use of a purified cellulase. More specifically, antiserum against the cellulase of the invention may be raised by immunizing rabbits (or other rodents) according to the procedure described by N. Axelsen *et al.* in: A Manual of Quantitative Immunoelectrophoresis, Blackwell Scientific Publications, 1973, Chapter 23, or A. Johnstone and R. Thorpe, Immunochemistry in Practice, Blackwell Scientific Publications, 1982 (more specifically pp. 27-31). Purified immunoglobulins may be obtained from the antisera, for example by salt precipitation ($(NH_4)_2 SO_4$), followed by dialysis and ion exchange chromatography, e.g. on DEAE-Sephadex. Immunochemical characterization of proteins may be done either by Outcherlony double-diffusion analysis (O. Ouchterlony in: Handbook of Experimental Immunology (D.M. Weir, Ed.), Blackwell Scientific Publications, 1967, pp. 655-706), by crossed immunoelectrophoresis (N. Axelsen et al., supra, Chapters 3 and 4), or by rocket immunoelectrophoresis (N. Axelsen et al., Chapter 2).

Media

YPD: 10 g yeast extract, 20 g peptone, H_2O to 900 ml. Autoclaved, 100 ml 20% glucose (sterile filtered) added.

YPM: 10 g yeast extract, 20 g peptone, H_2O to 900 ml. Autoclaved, 100 ml 20% maltodextrin (sterile filtered) added.

10 x Basal salt: 75 g yeast nitrogen base, 113 g succinic acid, 68 g NaOH, H_2O ad 1000 ml, sterile filtered.

SC-URA: 100 ml 10 x Basal salt, 28 ml 20% casamino acids without vitamins, 10 ml 1% tryptophan, H_2O ad 900 ml, autoclaved, 3.6 ml 5% threonine and 100 ml 20% glucose or 20% galactose added.

SC-URA agar: SC-URA, 20 g/l agar added.

PD agar: 39 g potato dextrose agar, DIFCO 0013; add deionized water up to 1000 ml; autoclave (121°C for 15-20 min).

PC agar: Potatoes and carrots (grinded, 20 g of each) and water, added up to 1000 ml, are boiled for 1 hr; agar (20 g/l of Merck 1614); autoclave (121°C for 20 min)

PC liquid broth: as PC agar but without the Agar

PD liquid broth: 24 g potato dextrose broth, Difco 0549, deionized water up to 1000 ml; autoclave (121°C for 15-20 min)

PC and PD liquid broth with cellulose: add 30 g Solcafloc (Dicacel available from Dicalite-Europe-Nord, 9000 Gent, Belgium) per 1000 ml

PB-9 liquid broth: 12 g Rofec (Roquette 101-0441) and 24 g glucose are added to 1000 ml water; pH is adjusted to 5.5; 5 ml mineral oil and 5 g $CaCo_3$ are added per 1000 ml. Autoclave (121°C for 40 min)

YPG liquid broth: 4 g yeast extract (Difco 0127), 1 g KH_2PO_4 (Merck4873), 0.5 g $MgSO_4.7H_2O$ Merck 5886, 15 g Dextrose, Roquette 101-0441, 0.1 ml Pluronic (101-3088); deionized water up to 1000 ml; autoclave (20 min at 121°C)

Dilute salt solution (DS): Make up two stock solutions:
P-stock: 13.61 g KH_2PO_4; 13.21 g $(NH_4)_2PO_4$, 17.42 g KH_2PO_4; deionized water up to 100 ml Ca/Mg stock: 7.35 g $CaCl_2$, $2H_2O$, 10.17 g $MgCl_2$, $6H_2O$, deionized water up to 100 ml; pH adjusted to 7.0; autoclaving (121°C; 20 min)

Mix 0.5 ml P-stock with 0.1 ml Ca/Mg stock
add deionized water up to 1000 ml
AZCL HE cellulose (Megazyme, Australia).

Uses

During washing and wearing, dyestuff from dyed fabrics or garment will conventionally bleed from the fabric which then looks faded and worn. Removal of surface fibers from the fabric will partly restore the original colours and looks of the fabric. By the term "colour clarification", as used herein, is meant the partly restoration of the initial colours of fabric or garment throughout multiple washing cycles.

The term "de-pilling" denotes removing of pills from the fabric surface.

The term "soaking liquor" denotes an aqueous liquor in which laundry may be immersed prior to being subjected to a conventional washing process. The soaking liquor may contain one or more ingredients conventionally used in a washing or laundering process.

The term "washing liquor" denotes an aqueous liquor in which laundry is subjected to a washing process, i.e. usually a combined chemical and mechanical action either manually or in a washing machine. Conventionally, the washing liquor is an aqueous solution of a powder or liquid detergent composition.

The term "rinsing liquor" denotes an aqueous liquor in which laundry is immersed and treated, conventionally immediately after being subjected to a washing process, in order to rinse the

laundry, i.e. essentially remove the detergent solution from the laundry. The rinsing liquor may contain a fabric conditioning or softening composition.

The laundry subjected to the method of the present invention may be conventional washable laundry. Preferably, the major part of the laundry is sewn or unsewn fabrics, including knits, wovens, denims, yarns, and toweling, made from cotton, cotton blends or natural or manmade cellulosics (e.g. originating from xylan-containing cellulose fibers such as from wood pulp) or blends thereof. Examples of blends are blends of cotton or rayon/viscose with one or more companion material such as wool, synthetic fibers (e.g. polyamide fibers, acrylic fibers, polyester fibers, polyvinyl alcohol fibers, polyvinyl chloride fibers, polyvinylidene chloride fibers, polyurethane fibers, polyurea fibers, aramid fibers), and cellulose-containing fibers (e.g. rayon/viscose, ramie, flax/linen, jute, cellulose acetate fibers, lyocell).

Detergent Compositions

According to one aspect of the present invention, the present endoglucanases may typically be components of a detergent composition. As such, they may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or protected enzymes. Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or nonaqueous.

The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cationic, or zwitterionic. The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzenesulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkanesulfonates

(SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid, or soap. It may also contain 0-40% of nonionic surfactant such as alcohol ethoxylate (AEO or AE), carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamine oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

The detergent composition may additionally comprise one or more other enzymes such as amylase, lipase, cutinase, protease, peroxidase, and oxidase, e.g. laccase.

The detergent may contain 1-65% of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst). The detergent may also be unbuilt, i.e. essentially free of detergent builder.

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose (CMC), poly(vinylpyrrolidone) (PVP), polyethyleneglycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent may contain a bleaching system which may comprise a H_2O_2 source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine (TAED) or nonanoyloxybenzenesulfonate (NOBS). Alternatively, the bleaching system may comprise peroxyacids of, e.g., the amide, imide, or sulfone type.

The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative such as, e.g., an aromatic borate ester, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as, e.g., fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, bactericides, optical brighteners, or perfume.

The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11.

Particular forms of detergent compositions within the scope of the invention include:

1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	7 - 12%

Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO) or alkyl sulfate (e.g. C_{16-18})	1 - 4%
Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na_2CO_3)	14 - 20%
Soluble silicate (as $Na_2O,2SiO_2$)	2 - 6%
Zeolite (as $NaA1SiO_4$)	15 - 22%
Sodium sulfate (as Na_2SO_4)	0 - 6%
Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
Sodium perborate (as $NaBO_3.H_2O$)	11 - 18%
TAED	2 - 6%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume, optical brightener, photobleach)	0 - 5%

2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	6 - 11%
Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO or alkyl sulfate (e.g. C_{16-18})	1 - 3%
Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na_2CO_3)	15 - 21%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaA1SiO_4$)	24 - 34%
Sodium sulfate (as Na_2SO_4)	4 - 10%
Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	5 - 9%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	7 - 14%
Soap as fatty acid (e.g. C_{16-22} fatty acid)	1 - 3%
Sodium carbonate (as Na_2CO_3)	10 - 17%
Soluble silicate (as $Na_2O,2SiO_2$)	3 - 9%
Zeolite (as $NaA1SiO_4$)	23 - 33%
Sodium sulfate (as Na_2SO4)	0 - 4%
Sodium perborate (as $NaBO_3.H_2O$)	8 - 16%
TAED	2 - 8%
Phosphonate (e.g. EDTMPA)	0 - 1%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume, optical brightener)	0 - 5%

4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	8 - 12%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	10 - 25%
Sodium carbonate (as Na_2CO_3)	14 - 22%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 5%
Zeolite (as $NaA1SiO_4$)	25 - 35%
Sodium sulfate (as Na_2SO_4)	0 - 10%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

5) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO or C_{12-15} alcohol, 5 EO)	12 - 18%
Soap as fatty acid (e.g. oleic acid)	3 - 13%

Alkenylsuccinic acid (C_{12-14})	0 - 13%
Aminoethanol	8 - 18%
Citric acid	2 - 8%
Phosphonate	0 - 3%
Polymers (e.g. PVP, PEG)	0 - 3%
Borate (as B_4O_7)	0 - 2%
Ethanol	0 - 3%
Propylene glycol	8 - 14%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener)	0 - 5%

6) An aqueous structured liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
Alcohol ethoxylate (e.g. C_{12-15} alcohol,7 EO, or C_{12-15}alcohol,5 EO)	3 - 9%
Soap as fatty acid (e.g. oleic acid)	3 - 10%
Zeolite (as $NaA1SiO_4$)	14 - 22%
Potassium citrate	9 - 18%
Borate (as B_4O_7)	0 - 2%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. PEG, PVP)	0 - 3%
Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0 - 3%
Glycerol	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners)	0 - 5%

7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Fatty alcohol sulfate	5 - 10%
Ethoxylated fatty acid monoethanolamide	3 - 9%
Soap as fatty acid	0 - 3%

Sodium carbonate (as Na_2CO_3)	5 - 10%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaA1SiO_4$)	20 - 40%
Sodium sulfate (as Na_2SO_4)	2 - 8%
Sodium perborate (as $NaBO_3.H_2O$)	12 - 18%
TAED	2 - 7%
Polymers (e.g. maleic/acrylic acid copolymer, PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, suds suppressors, perfume)	0 - 5%

8) A detergent composition formulated as a granulate comprising

Linear alkylbenzenesulfonate (calculated as acid)	8 - 14%
Ethoxylated fatty acid monoethanolamide	5 - 11%
Soap as fatty acid	0 - 3%
Sodium carbonate (as Na_2CO_3)	4 - 10%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaA1SiO_4$)	30 - 50%
Sodium sulfate (as Na_2SO_4)	3 - 11%
Sodium citrate (as $C_6H_5Na_3O_7$)	5 - 12%
Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

9) A detergent composition formulated as a granulate comprising

Linear alkylbenzenesulfonate (calculated as acid)	6 - 12%
Nonionic surfactant	1 - 4%
Soap as fatty acid	2 - 6%
Sodium carbonate (as Na_2CO_3)	14 - 22%
Zeolite (as $NaA1SiO_4$)	18 - 32%
Sodium sulfate (as Na_2SO_4)	5 - 20%
Sodium citrate (as $C_6H_5Na_3O_7$)	3 - 8%
Sodium perborate (as $NaBO_3.H_2O$)	4 - 9%

Bleach activator (e.g. NOBS or TAED)	1 - 5%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. polycarboxylate or PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, perfume)	0 - 5%

10) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 23%
Alcohol ethoxysulfate (e.g. C_{12-15} alcohol, 2-3 EO)	8 - 15%
Alcohol ethoxylate (e.g. C_{12-15} alcohol,7 EO, or C_{12-15}alcohol, 5 EO)	3 - 9%
Soap as fatty acid (e.g. lauric acid)	0 - 3%
Aminoethanol	1 - 5%
Sodium citrate	5 - 10%
Hydrotrope (e.g. sodium toluensulfonate)	2 - 6%
Borate (as B_4O_7)	0 - 2%
Carboxymethylcellulose	0 - 1%
Ethanol	1 - 3%
Propylene glycol	2 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0 - 5%

11) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	20 - 32%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	6 - 12%
Aminoethanol	2 - 6%
Citric acid	8 - 14%
Borate (as B_4O_7)	1 - 3%
Polymer (e.g. maleic/acrylic acid copolymer, anchoring polymer such as, e.g., lauryl methacrylate-/acrylic acid copolymer)	0 - 3%
Glycerol	3 - 8%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. hydrotropes, dispersants, perfume, optical	0 - 5%

brighteners)	

12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap)	25 - 40%
Nonionic surfactant (e.g. alcohol ethoxylate)	1 - 10%
Sodium carbonate (as Na_2CO_3)	8 - 25%
Soluble silicates (as Na_2O, $2SiO_2$)	5 - 15%
Sodium sulfate (as Na_2SO_4)	0 - 5%
Zeolite (as $NaA1SiO_4$)	15 - 28%
Sodium perborate (as $NaBO_3.4H_2O$)	0 - 20%
Bleach activator (TAED or NOBS)	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. perfume, optical brighteners)	0 - 3%

13) Detergent formulations as described in 1) - 12) wherein all or part of the linear alkylbenzenesulfonate is replaced by (C_{12}-C_{18}) alkyl sulfate.

14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C_{12}-C_{18}) alkyl sulfate	9 - 15%
Alcohol ethoxylate	3 - 6%
Polyhydroxy alkyl fatty acid amide	1 - 5%
Zeolite (as $NaA1SiO_4$)	10 - 20%
Layered disilicate (e.g. SK56 from Hoechst)	10 - 20%
Sodium carbonate (as Na_2CO_3)	3 - 12%
Soluble silicate (as Na_2O,$2SiO_2$)	0 - 6%
Sodium citrate	4 - 8%
Sodium percarbonate	13 - 22%
TAED	3 - 8%
Polymers (e.g. polycarboxylates and PVP=	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%

Minor ingredients (e.g. optical brightener, photo bleach, perfume, suds suppressors)	0 - 5%

15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C_{12}-C_{18}) alkyl sulfate	4 - 8%
Alcohol ethoxylate	11 - 15%
Soap	1 - 4%
Zeolite MAP or zeolite A	35 - 45%
Sodium carbonate (as Na_2CO_3)	2 - 8%
Soluble silicate (as $Na_2O,2SiO_2$)	0 - 4%
Sodium percarbonate	13 - 22%
TAED	1 - 8%
Carboxymethyl cellulose	0 - 3%
Polymers (e.g. polycarboxylates and PVP)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, phosphonate, perfume)	0 - 3%

16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified bleach systems.

17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.

18) Detergent compositions as described in 1), 3), 7), 9), 12), 14) and 15) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

19) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant such as, e.g., linear alkoxylated primary alcohol, a builder system (e.g. phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

The endoglucanase may be incorporated in concentrations conventionally employed in detergents. It is at present contemplated that, in the laundry composition of the invention, the

cellulase may be added in an amount corresponding to 0.0001-10 mg (calculated as pure enzyme protein) of cellulase per liter of wash liquor.

According to yet another aspect of the present invention, endoglucanase may typically be a component of a fabric conditioning or softener composition. Examples of conventional softener compositions are disclosed in e.g. EP 0 233 910.

Textile applications

In another embodiment, the present invention relates to use of the endoglucanase of the invention in the bio-polishing process. Bio-Polishing is a specific treatment of the yarn surface which improves fabric quality with respect to handle and appearance without loss of fabric wettability. The most important effects of Bio-Polishing can be characterized by less fuzz and pilling, increased gloss/luster, improved fabric handle, increased durable softness and altered water absorbency. Bio-Polishing usually takes place in the wet processing of the manufacture of knitted and woven fabrics. Wet processing comprises such steps as e.g. desizing, scouring, bleaching, washing, dying/printing and finishing. During each of these steps, the fabric is more or less subjected to mechanical action. In general, after the textiles have been knitted or woven, the fabric proceeds to a desizing stage, followed by a scouring stage, etc. Desizing is the act of removing size from textiles. Prior to weaving on mechanical looms, warp yarns are often coated with size starch or starch derivatives in order to increase their tensile strength. After weaving, the size coating must be removed before further processing the fabric in order to ensure a homogeneous and wash-proof result. It is known that in order to achieve the effects of Bio-Polishing, a combination of cellulytic and mechanical action is required. It is also known that "super-softness" is achievable when the treatment with a cellulase is combined with a conventional treatment with softening agents. It is contemplated that use of the endoglucanase of the invention for bio-polishing of cellulosic fabrics is advantageous, e.g. a more thorough polishing can be achieved. Bio-polishing may be obtained by applying the method described e.g. in WO 93/20278.

Stone-washing

It is known to provide a "stone-washed" look (localized abrasion of the colour) in dyed fabric, especially in denim fabric or jeans, either by washing the denim or jeans made from such fabric in the presence of pumice stones to provide the desired localized lightening of the colour of the fabric or by treating the fabric enzymatically, in particular with cellulytic enzymes. The treatment with an endoglucanase of the present invention may be carried out either alone such as disclosed in US

4,832,864, together with a smaller amount of pumice than required in the traditional process, or together with perlite such as disclosed in WO 95/09225.

Pulp and paper applications

In the papermaking pulp industry, the endoglucanase of the present invention may be applied advantageously e.g. as follows:

- For debarking: pretreatment with the endoglucanase may degrade the cambium layer prior to debarking in mechanical drums resulting in advantageous energy savings.

- For defibration: treatment of a material containing cellulosic fibers with the endoglucanase prior to refining or beating may result in reduction of the energy consumption due to the hydrolysing effect of the cellulase on the interfibre surfaces. Use of the endoglucanase may result in improved energy savings as compared to the use of known enzymes, since it is believed that the enzyme composition of the invention may possess a higher ability to penetrate fibre walls.

- For fibre modification, i.e. improvement of fibre properties where partial hydrolysis across the fibre wall is needed which requires deeper penetrating enzymes (e.g. in order to make coarse fibers more flexible). Deep treatment of fibers has so far not been possible for high yield pulps e.g. mechanical pulps or mixtures of recycled pulps. This has been ascribed to the nature of the fibre wall structure that prevents the passage of enzyme molecules due to physical restriction of the pore matrix of the fibre wall. It is contemplated that the present endoglucanase is capable of penetrating into the fibre wall.

- For drainage improvement. The drainability of papermaking pulps may be improved by treatment of the pulp with hydrolysing enzymes, e.g. cellulases. Use of the present endoglucanase may be more effective, e.g. result in a higher degree of loosening bundles of strongly hydrated micro-fibrils in the fines fraction (consisting of fibre debris) that limits the rate of drainage by blocking hollow spaces between fibers and in the wire mesh of the paper machine. The Canadian standard freeness (CSF) increases and the Schopper-Riegler drainage index decreases when pulp in subjected to cellulase treatment, see e.g. US patent 4,923,565; TAPPI T227, SCAN C19:65.ence.

- For inter fibre bonding. Hydrolytic enzymes are applied in the manufacture of papermaking pulps for improving the inter fibre bonding. The enzymes rinse the fibre surfaces for impurities e.g. cellulosic debris, thus enhancing the area of exposed cellulose with attachment to the fibre wall, thus improving the fibre-to-fibre hydrogen binding capacity. This process is also referred to as dehornification. Paper and board produced with a cellulase containing enzyme preparation may have an improved strength or a reduced grammage, a smoother surface and an improved printability.

- For enzymatic deinking. Partial hydrolysis of recycled paper during or upon pulping by use of hydrolysing enzymes such as cellulases are known to facilitate the removal and agglomeration of ink particles. Use of the present endoglucanse may give a more effective loosening of ink from the surface structure due to a better penetration of the enzyme molecules into the fibrillar matrix of the fibre wall, thus softening the surface whereby ink particles are effectively loosened. The agglomeration of loosened ink particles are also improved, due to a more efficient hydrolysis of cellulosic fragments found attached to ink particles originating from the fibres.

The treatment of lignocellulosic pulp may, e.g., be performed as described in WO 91/14819, WO 91/14822, WO 92/17573 and WO 92/18688.

Degradation of plant material

In yet another embodiment, the present invention relates to use of the endoglucanase and/or enzyme preparation according to the invention for degradation of plant material e.g. cell walls.

It is contemplated that the novel endoglucanase and/or enzyme preparation of the invention is useful in the preparation of wine, fruit or vegetable juice in order to increase yield. Endoglucanases according to the invention may also be applied for enzymatic hydrolysis of various plant cell-wall derived materials or waste materials, e.g. agricultural residues such as wheat-straw, corn cobs, whole corn plants, nut shells, grass, vegetable hulls, bean hulls, spent grains, sugar beet pulp, and the like. The plant material may be degraded in order to improve different kinds of processing, facilitate purification or extraction of other components like purification of beta-glucan or beta-glucan oligomers from cereals, improve the feed value, decrease the water binding capacity, improve the degradability in waste water plants, improve the conversion of e.g. grass and corn to ensilage, etc.

The following examples illustrate the invention.

EXAMPLE 1

Cellulytic enzymes from 4 fungi, belonging to 3 families under two orders within the Ascomycetes were detected by expression cloning; corresponding DNA sequences were determined; the enzymes heterologously expressed, and produced by liquid fermentation, characterized and demonstrated to give good performance in colour clarification assays.

Isolate CBS 117.65, CBS 478.94, NRRL 8126, and ATCC 10523 were grown in shake flask cultures on cellulose enriched potato dextrose broth, incubated for 5 days at 26°C (shaking conditions, 150 rpm).

A. Cloning and expression of an endoglucanase from *Myceliophthora thermophila*, *Acremonium sp.*, and *Thielavia terrestris* and *Volutella colletotrichoides*

mRNA was isolated from *Myceliophthora thermophila, Acremonium sp., Thielavia terrestris* and *Volutella colletotrichoides*, respectively, grown in a cellulose-containing fermentation medium with agitation to ensure sufficient aeration. Mycelia were harvested after 3-5 days' growth, immediately frozen in liquid nitrogen and stored at -80□C. Libraries from *Myceliophthora thermophila, Acremonium sp., Thielavia terrestris* and *Volutella colletotrichoides*, respectively, each consisting of approx. 10^6 individual clones were constructed in *E. coli* as described with a vector background of 1%.

Plasmid DNA from some of the pools from each library was transformed into yeast, and 50-100 plates containing 250-400 yeast colonies were obtained from each pool.

Endoglucanase-positive colonies were identified and isolated on SC-agar plates with the AZCL HE cellulose assay. cDNA inserts were amplified directly from the yeast colonies and characterized as described in the Materials and Methods section above.

The DNA sequence of the cDNA encoding the endoglucanase from *Myceliophthora thermophila* is of SEQ ID NO: 1 and the corresponding amino acid sequence is also of SEQ ID NO: 2. The cDNA is obtainable from the plasmid in DSM 9770.

The DNA sequence of the cDNA encoding the endoglucanase from *Acremonium sp.* is of SEQ ID NO: 7 and the corresponding amino acid sequence is of SEQ ID NO: 8. The cDNA is obtainable from the plasmid in DSM 10082.

The DNA sequence of the cDNA encoding the endoglucanase from *Thielavia terrestris* is of SEQ ID NO: 11 and the corresponding amino acid sequence is of SEQ ID NO: 12. The cDNA is obtainable from the plasmid in DSM 10081.

The DNA sequence of the cDNA encoding the endoglucanase from *Volutella colletotrichoides* is of SEQ ID NO: 21 and the corresponding amino acid sequence is of SEQ ID NO: 22. The cDNA is obtainable from the plasmid in DSM 10571.

Total DNA was isolated from a yeast colony and plasmid DNA was rescued by transformation of *E. coli* as described above. In order to express the endoglucanases in *Aspergillus*, the DNA was digested with appropriate restriction enzymes, size fractionated on gel, and a fragment corresponding to the endoglucanase gene from *Myceliophthora thermophila, Acremonium sp., Thielavia terrestris* and *Volutella colletotrichoides*, respectively, was purified. The genes were subsequently ligated to pHD414, digested with appropriate restriction enzymes, resulting in the plasmids pA2C193, pA2C357, pA2C385 and pA2C488, respectively.

After amplification of the DNA in *E. coli* the plasmids were transformed into *Aspergillus oryzae* as described above.

Test of *A. oryzae* transformants

Each of the transformants were tested for endoglucanase activity as described above. Some of the transformants had endoglucanase activity which was significantly larger than the *Aspergillus oryzae* background. This demonstrates efficient expression of the endoglucanases in *Aspergillus oryzae*. The transformants with the highest endoglucanase activity were selected and inoculated in a 500 ml shake flask with YPM media. After 3-5 days of fermentation with sufficient agitation to ensure good aeration, the culture broth was centrifuged for 10 minutes at 2000 g and the supernatant recovered.

B. Determination of endoglucanase activity

The cellulytic activity of the endoglucanase may be determined relative to an analytical standard and expressed in the unit S-CEVU.

Cellulytic enzymes hydrolyse CMC, thereby decreasing the viscosity of the incubation mixture. The resulting reduction in viscosity may be determined by a vibration viscosimeter (e.g. MIVI 3000 from Sofraser, France).

Determination of the cellulytic activity, measured in terms of S-CEVU, may be determined according to the analysis method AF 301.1 which is available from the Applicant upon request.

The S-CEVU assay quantifies the amount of catalytic activity present in the sample by measuring the ability of the sample to reduce the viscosity of a solution of carboxy-methylcellulose (CMC). The assay is carried out at 40°C, pH 7.5 using a relative enzyme standard for reducing the viscosity of the CMC substrate.

Assay for determination of endoglucanase activity in terms of SAVI units using phosphoric-acid swollen cellulose (PASC):

Definition: 1 SAVI-U is the amount of enzyme which forms an amount of reducing carbohydrates equivalent to 1 μmol of glucose per minute.

Assay condition:

Enzyme solution: 0.5 ml

4 g/l PASC in 0,1 M Buffer: 2.0 ml

20 min, 40°C

Sensitivity:

Max 0.1 SAVIU/ml = approx. 1 S-CEVU/ml (CMC viscosity)

Min 0.01 SAVIU/ml = approx. 0.1 S-CEVU/ml

Determination of formation of reducing sugars:

The reducing groups assay was performed according to Lever, M. A new reaction for colormetric determination of carbohydrates. Anal. Biochem. 1972. Vol 47 (273-279). Reagent mixture was prepared by mixing 1,5 gram p-hydroxybenzoic-acide hydracide (PHBAH) with 5 gram sodium tartrate in 100 ml 2 % sodium hydroxide.

Substrate:

PASC stock solution was prepared the following way using ice cold acetone and phosphoric acid. 5 gram of cellulose (Avicel®) was moistered with water, and 150 ml ice cold 85% ortho-phosphoric acid was added. The mixture was placed in ice bath under slow stirring for 1 hr. Then 100 ml ice cold acetone was added with stirring. The slurry was transferred to a Buchner filter with pyrex sintered disc number 3 and then washed three times with 100 ml ice cold acetone, and sucked as dry as possible after each wash. Finally, the filter cake was washed twice with 500 ml water, sucked as dry as possible after each wash. The PASC was mixed with deionized water to a total volume of 300 ml, blended to homogeneity (using the Ultra Turrax Homogenizer) and stored in refrigerator (up to one month).

Substrate equilibration with buffer: 20 gram phosphoric acid swollen cellulose PASC stock solution was centrifuged for 20 min at 5000 rpm., the supernatant was poured of; the sediment was resuspended in 30 ml of buffer and centrifuged for 20 min. at 5000 rpm., the supernatant was poured of, and the sediment was resuspended in buffer to a total of 60 g corresponding to a substrate concentration of 5 g cellulose/litre.

Buffer for pH 8.5 determination: 0.1 M Barbital.

Buffer for pH 10 determination: 0.1 M Glycine.

Procedure:

1. Dilution of enzyme samples

   The enzyme solution is diluted in the same buffer as the substrate.

2. Enzyme reaction

The substrate in buffer solution is preheated for 5 min. at 40°C (2 ml). Then the enzyme solution (diluted to between 0.2 and 1 S-CEVU/ml) 0.5 ml is added and mixed for 5 sec. Enzymes blanks are obtained by adding the stop reagent before enzyme solution. Incubate for 20 min. at 40°C. The reaction is stopped by adding 0.5 ml 2% NaOH solution and mixing for 5 sec.

The samples are centrifuged for 20 min. at 5000 rpm. 1 ml supernatant is mixed with 0.5 ml PHBAH reagent and boiled for 10 min. The test tubes are cooled in an ice water bath.

3. Determination of reducing end groups:

The absorbancy at 410 nm is measured using a spectrophotometer. Blanks are prepared by adding sodium hydroxide before adding enzyme solution.

A standard glucose curve was obtained by using glucose concentrations of 5, 10, 15 and 25 mg/l in the same buffer and adding PHBAH reagent before boiling. The release of reducing glucose equivalent is calculated using this standard curve.

4. Calculation of catalytic activity:

Measure absorbance at 410 nm

1) Standard curve

(Glucose) - (H_2O) vs concentration of glucose

2) Enzyme sample

(Sample) - (Blank)

Calculate glucose concentration according to a standard curve

Activity (SAVIU/ml):

$$\frac{\text{X (mg glucose/l)} * \text{Dilution}}{\text{180.16 (MW of glucose)} * \text{20 (min)}}$$

C. Purification and characterisation of the endoglucanase from *M. thermophila*

Aspergillus oryzae transformed with pA2C193 was grown on YPM medium for 4 days. The liquid was then centrifuged and sterile filtered.

The sample was concentrated by ultrafiltration on AMICON cells using a DOW membrane GR61PP with cut-off 20 kD. The Uf-concentrate was analyzed for S-CEVU/ml and SaviU/ml with the following result:

UF-concentrate	S-CEVU/ml	SaviU/ml
9.25 ml	570	41

Purification:

2 ml of the UF-concentrate was diluted 5 times to lower the ionic strength and filtered through 0.22 μm disk filter. This sample was applied to a Mono Q® HR5/5 Pharmacia column, equilibrated with 50 mM Tris/HCl buffer, pH 7.5, (buffer A) and a flow of 1 ml/min. After wash to

baseline, with buffer A, the column was eluted with a Tris/HCl buffer, pH 7.5, containing 1 M NaCl (buffer B), the elution gradient was 0-50% buffer B in 1 hour.

After 36 min. a peak complex showed up, 1 ml fractions were picked up and the first 10 fractions showed cellulase activity on CMC/Agarose/congo-red plates.

These fractions were pooled and concentrated, by ultrafiltration on AMICON cells using a DOW membrane GR61PP with cut-off 20 kD, to 3 ml.

This sample was applied to a HiLoad 26/60 Superdex 75™ prep grade Pharmacia column, equilibrated with 100 mM Na-Acetate buffer, pH 6.35, and a 1 ml/min flow.

After 82 min. a peak showed up, 1 ml fractions were picked up and the first 10 fractions showed cellulase activity on CMC/Agarose/congo-red plates.

These fractions were pooled and the following results were obtained:

A_{280}=0.15

A_{280}/A_{260}=1.62

Mw(SDS)=22 kD

pI=3.5 - 5

Purity on SDS-PAGE =100%

S-CEVU/ml=28.5

S-CEVU/A_{280}=188

S-CEVU/mg=436

Extinction coefficient=54880 (calculated)

Mw(calculated)=22 kD

The Extinction coefficient is based on the content of tyrosine, tryptophane and cystein calculated from the sequence of SEQ ID NO: 2 (the amino acid sequence). SDS-Page was performed on NOVEX Pre-Cast Gels 4-20% Tris-Glycine Gel 1.0 mm x 10 Well.

IEF was performed on Pharmacia PAGplate pH 3.5 - 9.5, the activity was visualized by CMC-Congored overlaying.

Determination of K_M & k_{cat}:

k_m and k_{cat} was determined in the same manner as the determination of SAVI Units at pH 8.5 with a substrate concentration up to 8 g/l.

The following results were obtained:

k_{cat} 38 per sec.

k_m 5 g/l,

phosporic acid swollen cellulose, pH 8.5.

Specific activity on CMC at pH 7.5:

436 S-CEVU per mg protein.

D. Determination of pH and temperature profile of the endoglucanase from *M. thermophila*

The pH profile was determined at the following conditions:

Buffers of pH values between 2.5 and 10.0 were made by mixing 0.1 M Tri-sodium phosphate with 0.1 M citric acid. Purified endoglucanase was diluted to ensure the assay response to be within the linear range of the assay. The substrate was a 0.4% suspension of AZCL-HE-cellulose (MegaZyme) mixed 1:1 with the citrate/phosphate buffer to a final substrate concentration of 0.2% AZCL-HE-cellulose. 1 ml substrate in Eppendorf® 1.5 ml polypropylene tubes were added 10 µl of enzyme solution and incubated for 15 minutes in Eppendorf® temperature controlled Thermomixers before heat-inactivation of enzymes for 20 minutes at 95°C in a separate Thermomixer. The tubes were centrifuged and 200 µl of each supernatant was transferred to a well in a 96 well microtiter plate and OD was measured at 620 nm in an ELISA reader (Labsystems Multiskan® MCC/340).

For the pH optimum incubations took place at 30°C. For each pH value, three tubes were added enzyme and incubated before heat-inactivation, whereas one tube (the blank) was added enzyme and heat-inactivated immediately. The mean value of the three incubated samples was calculated and the blank value was substracted.

The following pH profile was determined:

PH	Relative Activity
2.5	<10%
3	<10%
3.5	22%
4	87%
4.5	89%
5	100%
6	94%
6.5	86%
7	78%
7.5	73%
8	68%
8.5	54%

9	31%
10	18%

It is seen that the endoglucanase has more than 60% activity between pH 4.0 and 8.0 and optimal activity at pH 5.0-6.0.

Temperature profile:

The temperature optimum was determined in the same manner at pH 5.5. The temperatures ranged from 30°C to 80°C. For each temperature three incubations were carried out and the mean calculated. Three blanks were produced by immediate heat-inactivation of enzyme and the mean was subtracted from the incubated sample values.

It is seen that the endoglucanase has optimal activity at 50-70°C.

Temp. (°C)	30	40	50	60	70	80
Relative Activity	74%	77%	99%	100%	93%	62%

The temperature stability was determined in the same manner at pH 5.5 and 30°C, and, further, the enzyme solutions were preheated for 1 hour at the actual temperature and cooled on ice. The residual activity is shown below in % of the activity of a non-preheated enzyme sample:

Temp. (°C)	40	50	60	70	80
Relative Activity	95%	84%	92%	86%	24%

E. Color clarification of Myceliophthora cellulase (SEQ ID NO: 2) measured as removal of surface fibrils and fibers protruding from the yarn of a textile containing cellulosic fibers

Apparatus	:	Terg-o-tometer
Liquid volume	:	100 ml
Agitation	:	150 movements/min with vertical stirrer
Rinse time	:	5 min in tapwater
Washing temp	:	40°C
Washing liqour	:	0.05 M phosphate buffer
pH	:	7.0
Washing time	:	30 min
Repetitions	:	2
Enzymes	:	Myceliophthora SEQ ID NO: 2

Dosage	:	500 and 2500 S-CEVU/l
Textile	:	2 swatches of aged black 100% cotton 5x6 cm (0.9 gram)
Drying	:	Tumble dry
Evaluation	:	The light remission is measured by a Datacolor Elrepho Remission spectrophotometer. Remission is calculated as delta L (Hunter Lab-values). When the surface fibrils and fibers protruding from the yarn are removed by the cellulase, the surface of the black fabric appears darker, and lower L values are obtained.

The sample is compared with a blind sample, i.e. washed without enzyme:

No cellulase	500 ECU/l	2500 ECU/l
0.00	-1.41	-1.91

Delta L-values compared to blind sample.

The data shows that Myceliophthora cellulase without CBD gives good color clarification under the conditions tested.

F. Construction of the gene fusions between the endoglucanase from *Myceliophthora thermophila* and the 43 kD endoglucanase from *Humicola insolens*

The purpose of the two constructions was to make derivatives of the endoglucanase from *M. thermophila* with the linker and CBD from the 43 kD endoglucanase from *H. insolens* (disclosed in WO 91/17243). The native endoglucanase from *M. thermophila* do not have a linker and/or a cellulose binding domain, CBD.

CM1: Construction 1 consists of the endoglucanase from *M. thermophila* (225 amino acids) and the 72 C-terminal amino acids from the *H. insolens* 43 kD endoglucanase.

CM2: Construction 2 consists of the endoglucanase from *M. thermophila* (225 amino acids) and the 83 C-terminal amino acids from the *H. insolens* 43 kD endoglucanase.

The 43 kD endoglucanase cDNA from *H. insolens* was cloned into pHD414 in such a way that the endoglucanase gene was transcribed from the Taka-promoter. The resulting plasmid was named pCaHj418.

In a similar way the cDNA encoding the endoglucanase from *M. thermophila* was cloned into pHD414 and the resulting plasmid was named pA2C193.

Primers:

primer 1:

5'- CGGAGCTCACGTCCAAGAGCGGCTGCTCCCGTCCCTCCAGCAGCACCAGCTCTCCGG -3' (SEQ ID NO: 88)

primer 2:

5'- CCGGAGAGCTGGTGCTGCTGGAGGGACGGGAGCAGCCGCTCTTGGACGTGAGCTCCG -3' (SEQ ID NO: 89)

primer 3:

5'- CGGAGCTCACGTCCAAGAGCGGCTGCTCCCGTAACGACGACGGCAACTTCCCTGC CG -3' (SEQ ID NO: 90)

primer 4:

5'- CGGCAGGGAAGTTGCCGTCGTCGTTACGGGAGCAGCCGCTCTTGGACGTGAGCTCCG -3' (SEQ ID NO: 91)

Taka-pro. primer: 5'- CAACATCACATCAAGCTCTCC -3' (SEQ ID NO: 92)

AMG-term. primer: 5'- CCCCATCCTTTAACTATAGCG -3' (SEQ ID NO: 93)

The endoglucanase fusions were constructed by the PCR overlap-extension method as described by Higuchi et al. 1988.

Construction 1:

Reaction A: The Polymerase Chain Reaction (PCR) was used to amplify the fragment of pCaHj418 between primer 1 and AMG-term. primer (the linker and CBD from the 43 kD endoglucanase from *H. insolens*).

Reaction B: PCR amplification of the fragment between Taka-pro. primer and primer 2 in pA2C193, the endoglucanase gene from M.thermophila.

Reaction C: The two purified fragments were used in a third PCR in the presence of the primers flanking the total region, i.e. Taka-pro. primer and AMG-term. primer.

Construction 2:

The same procedure was used where primer 3 and primer 4 had replaced respectively primer 1 and primer 2.

The fragment amplified in reaction C was purified, digested with restriction enzymes Xba I and BsstE II. The purified digested fragment was ligated into pA2C193 digested with restriction enzymes Xba I and BsstE II.

Competent cells from *E. coli* strain DH5αF' (New England Biolabs.) were transformed with the ligated plasmid and colonies containing the gene fusion were isolated. The sequence of the cloned part was verified by DNA sequencing.

The sequence of the gene in the two constructs are of SEQ ID NO: 3 and SEQ ID NO: 5.

Polymerase Chain Reactions were carried out under standard conditions, as recommended by Perkin-Elmer.

Reaction A and B started with 2 min. at 94°C followed by 20 cycles of (30 sec. at 94°C, 30 sec. at 50°C and 1 min. at 72°C) and end with 4 min. at 72°C.

Reaction C started with (2 min. at 94°C, 1 min. at 52°C and 2 min. at 72°C), followed by 15 cycles of (30 sec. at 94°C, 30 sec. at 52°C and 90 sec. at 72°C) and end with 4 min. at 72°C.

The two constructs were transformed into *Aspergillus oryzae* as described above.

G. Purification and characterisation of cloned cellulases with cellulose binding domains:

The cloned product is recovered after fermentation by separation of the extracellular fluid from the production organism.

About one gram of cellulase is then highly purified by affinity chromatography using 150 gram of Avicel in a slurry with 20 mm Sodium- phosphate pH 7.5.

The Avicel is mixed with the crude fermentation broth, which contains total about 1 gram of cellulase. After mixing at 4°C for 20 min the Avicel enzyme is packed into a column with a dimension of 50 times 200 mm about 400 ml total.

The column is washed with the 200 ml buffer, then washed with 0.5 M NaCl in the same buffer until no more protein elutes. Then washed with 500 ml 20 mm Tris pH 8.5. Finally the pure full length enzyme is eluted with 1% triethylamine pH 11.8.

The eluted enzyme solution is adjusted to pH 8 and concentrated using a Amicon cell unit with a membrane DOW GR61PP (polypropylene with a cut off of 20 KD) to above 5 mg protein per ml.

The purified cellulases were characterised as follow:

	Mw SDS-PAGE	pI	Molar E.280	S-CEVU per A.280
Myceliophthora (SEQ ID NO: 4)	43 kD	4	74.950	135
Acremonium (SEQ ID NO: 8)	40 kD	5	68.020	185
Thielavia (SEQ ID NO: 12)	35 kD	4.3	52.470	75

	pH Activity above 50%	N-terminal	Thermostability DSC
Myceliophthora (SEQ ID NO: 4)	5.0-9.0	Blocked.	80°C
Acremonium (SEQ ID NO: 8)	6.0-9.5	Blocked.	61°C
Thielavia (SEQ ID NO: 12)	5.0-9.0	ASGSG---	83°C

The purified cellulases was analysed for MW by SDS-PAGE and using standard LMW protein marker kit from Pharmacia the MW was calculated for the cellulases. The MW is apparently higher than the MW of the composition of the coding amin acids and is due to the fact the linker region are O-glycosylated resulting in this higher MW. The pI was determined using a Pharmacia Ampholine PAG plates pH 3.5 to 9.5 and again using a Pharmacia kit with known pI proteins.

The molar extinction coefficient was calculated based on the amin acids composition using the known absorbance of Tryptophan, Tyrosine and Cystein.

pH activity profile was obtained using CMC substrate, incubation for 20 min at 40°C at a 0.5 pH interval and measuring the formation of reducing sugars. The relative activity at the different pH was calculated and the table contains the interval with more than 50% relative activity has been measured.

The N-terminal was determined for the purified cellulase using a Applied Biosystems model 473A sequencer. The protein sequenceer was run according to the manufacturer instructions.

Two of the cellulases were blocked, this is due to the N-terminal glutamine which forms a pyroglutamate which can not be detected and which blocks further sequencing.

DSC Differential scanning calometry was done at neutral pH (7.0) using a MicroCalc Inc. MC calorimeter with a constant scan rate and raising the temperature from 20 to 90°C at a rate of 90° per hour.

Raising antibody. The cellulases from *Myceliophthora*, *Acremonium* and *Thielavia* were used for raising antibody in rabbits. 0.1 mg of the purified cellulase in 0.9 % NaCl solution mixed with Freunds adjuvant immediately prior to injection. The rabbits were immunized 10 times with one week interval. The immunoglobulin G fraction (IgG) was purified by ammonium sulfate precipitation (25% saturation). The precipitate was solubilized in water and then dialyzed extensively against sodium acetate buffer (pH 5.0, 50 mM) altering with deionized water. After filtration, the IgG fraction was stabilized with sodium azide (0.01%).

Using immunodiffusion in agar plates all three cellulases form a single immunoprecipitate with its homologous antiserum and no precipitate was seen between the 3 cloned cellulases and the sera raised against the other two cellulases.

H-I. Performance of endoglucanase of construction 1 (SEQ ID NO: 3) measured in buffer as removal of surface fibrils and fibers protruding from the yarn of a textile containing cellulosic fibers

Apparatus	:	Terg-o-tometer
Liquid volume	:	100 ml
Agitation	:	150 movements/min (rpm)
Rinse time	:	5 min in tap water
Washing temp	:	40°C
Water Hardness	:	1 mM $CaCl_2$
Washing liquor	:	0.05 M phosphate buffer
pH	:	7.0
Washing time	:	30 min
Repetitions	:	2
Textile	:	2 swatches of aged black, 100% cotton 5x6 cm
Drying	:	Tumble dry

Evaluation:

The light remission was measured by a Macbeth Color Eye 7000 Remission spectrophotometer. Remission is calculated as delta L (Hunter Lab-values). When the surface

fibrils and fibers protruding from the yarn were removed by the cellulase, the surface appeared more bright, and lower L values were obtained.

Results:

S-CEVU/l	0	250	1000
Inventive enzyme	0	-1.4	-1.6

The data show that the enzyme of the invention gives very good color clarification under the conditions tested.

H-II. Performance of cloned endoglucanase from *Thielavia terrestris* (SEQ ID NO: 12) in buffer measured as removal of surface fibrils and fibers protruding from the yarn of a textile containing cellulosic fibers

Apparatus	:	Terg-o-tometer
Liquid volume	:	100 ml
Agitation	:	150 movements/min with vertical stirrer
Rinse time	:	10 min in tapwater
Washing temp	:	40°
Washing liqour	:	0.05 M phosphate buffer.
pH	:	7.0
Washing time	:	30 min
Repetitions	:	2
Textile	:	2 swatches of aged black cotton 5x6 cm (app. 150 g/m^2)
Drying	:	Tumble dry

Evaluation:

The light remission was measured by a Datacolor Elrepho Remission spectrophotometer. Remission is calculated as delta L (Hunter Lab-values). When the surface fibrils and fibers protruding from the yarn are removed by the cellulase, the surface of the black fabric appears darker and nicer, and lower L values are obtained.

Results:

S-CEVU/l	0	50	200
Inventive enzyme	0	-0.66±0.10	-1.32±0.06

The data show that the cellulase gives good color clarification under the conditions tested.

H-III. Performance of endoglucanase of *Volutella colletrichoides* (SEQ ID NO: 9) measured in buffer as removal of surface fibrils and fibers protruding from the yarn of a textile containing cellulosic fibers

Apparatus	:	Terg-o-tometer
Liquid volume	:	100 ml
Agitation	:	150 movements/min with vertical stirrer
Rinse time	:	5 min in tapwater
Washing temp	:	40°
Washing liqour	:	0.05 M phosphate buffer
pH	:	7.0
Washing time	:	30 min
Repetitions	:	2
Dosage	:	2.5 S-CEVU/ml
Textile	:	2 swatches of aged black 100% cotton 5x6 cm (0.9 gram)
Drying	:	Tumble dry

Evaluation:

The light remission is measured by a Datacolor Elrepho Remission spectrophotometer. Remission is calculated as delta L (Hunter Lab-values). When the surface fibrils and fibers protruding from the yarn are removed by the cellulase, the surface of the black fabric appears darker, and lower L values are obtained.

The sample is compared with a blind sample, i.e. washed without enzyme:

No cellulase	With cellulase
0.00	-0.57

Delta L remission values compared to blind sample.

The data shows that the *Volutella colletrichoides* cellulase gives good color clarification under the conditions tested.

H-IV. Performance of cloned cellulases from *Thielavia terrestris* and *Acremonium sp.* CBS 478.94 in high pH heavy duty detergent measured as removal of surface fibrils and fibers protruding from the yarn of a textile containing cellulosic fibers

Apparatus	:	Terg-o-tometer
Liquid volume	:	150 ml
Agitation	:	150 movements/min with vertical stirrer

Rinse time	:	10 min in tapwater
Washing temp	:	35°C
Washing liqour	:	1.0 g/l US type HDG
		(zeolite/soda built, anionic/nonionic weight ratio > 2.5)
pH	:	10.0
Hardness	:	1.0 mM $CaCl_2$
		0.34 mM $MgCl_2$
Washing time	:	12 min
Repetitions	:	6
Textile	:	2 swatches of aged black cotton 5x6 cm (app. 150 g/m^2)
		2 swatches of heavy knitted cotton 5x6 cm (app. 600 g/m^2)
Drying	:	Tumble dry

Evaluation:

The light remission is measured by a Datacolor Elrepho Remission spectrophotometer. Remission is calculated as delta L (Hunter Lab-values). When the surface fibrils and fibers protruding from the yarn are removed by the cellulase, the surface of the black fabric appears darker and nicer, and lower L values are obtained. Different dosages of cloned cellulases from *Thielavia terrestris* (SEQ ID NO: 12) and *Acremonium sp.* CBS 478.94 (SEQ ID NO: 8), respectively, (denoted A and B, respectively) were tested.

Results:

S-CEVU/l	0	500	2000
A	0	-2.09±0.22	-2.86±0.19
B	0	-0.60±0.36	-1.96±0.23

The data show that both cellulases gives good color clarification under the conditions tested.

H-V. Performance of cellulases cloned from *Thielavia terrestris* and *Acremonium sp.* CBS 478.94, and construction 1 (SEQ ID NO: 3) measured as removal of surface fibrils and fibers protruding from the yarn of a textile containing cellulosic fibers

Apparatus	:	Terg-o-tometer
Liquid volume	:	150 ml
Agitation	:	150 movements/min with vertical stirrer
Rinse time	:	10 min in tapwater

Washing temp	:	35°C
Hardness	:	1.0 mM $CaCl_2$
		0.34 mM $MgCl_2$
Washing liqour	:	2.0 g/l HDL (neutral, citrate built HDL, with nonionic/anionic weight ration > 0.5)
pH	:	7.5
Washing time	:	30 min
Repetitions	:	2
Textile	:	2 swatches of aged black cotton 5x6 cm (app. 150 g/m^2)
		2 swatches of heavy knitted cotton 4x7 cm (app. 600 g/m^2)
Drying	:	Tumble dry

Evaluation:

The light remission is measured by a Datacolor Elrepho Remission spectrophotometer. Remission is calculated as delta L (CIE Lab-values). When the surface fibrils and fibers protruding from the yarn are removed by the cellulase, the surface of the black fabric appears darker and nicer, and lower L values are obtained. Three different dosages of cloned cellulases from *Thielavia terrestris* (SEQ ID NO: 12) and *Acremonium sp.* CBS 478.94 (SEQ ID NO: 8) and the construction 1 (SEQ ID NO: 3), respectively, (denoted A and B and C, respectively) were tested.

Results:

S-CEVU/l	0	100	200	400
A	0	-3.06±0.24	-3.15±0.27	-3.92±0.26
B	0	-1.75±0.27	-3.08±0.32	-3.51±0.44
C	0	-1.84±0.39	-1.70±0.47	-2.30±0.61

The data show that all cellulases give very good color clarification under the conditions tested.

I. Application of endoglucanases from Thielavia terrestris, Acremonium sp. and construction 1 (SEQ ID NO: 3) in denim finishing

Experimental

Apparatus: Washing machine Wascator FL 120

Liquid volume : 20 L

Fabric : 1.1 kg denim fabric, 14½ oz 100 % cotton

Desizing : 10 min, 55□C, pH 7
50 ml Aquazyme 120L
2.5 g/l Phosphate buffer

Abrasion : 2 hours;

pH and temperature varied according to the following table:

Enzyme SEQ ID	Activity	pH/temp	Buffer system
No. 3	1400 S-CEVU/g	6/55°C	2.5 g/l phosphate buffer
No. 12	292 S-CEVU/g	5/65°C	1 g/l citrate buffer
No. 8	782 S-CEVU/g	7/45°C	2.5 g/l phosphate buffer

Inactivation: 15 min, 80°C
1 g/l sodium carbonate

Rinses: Three rinse cycles of 5 min in cold tap water

Evaluation:

Abrasion: The remission from the fabric was determined at 420 nm using a Texflash 2000 as a measure of the abrasion level.

The results from the treatment of the denim fabric with different endoglucanases of the invention is shown in the following table:

Enzyme	Dosage	Trial conditions	Abrasion 420 nm
Blank	0 S-CEVU/g textile	pH 6, 55°C	9.96
SEQ ID NO: 3	10 S-CEVU/g textile	pH 6, 55°C	14.37
Blank	0 S-CEVU/g textile	pH 5, 65°C	9.26
SEQ ID NO: 12	10 S-CEVU/g textile	pH 5, 65°C	16.86
Blank	0 S-CEVU/g textile	pH 7, 45°C	9.47
SEQ ID NO: 8	10 S-CEVU/g textile	pH 7, 45°C	14.08

All tested cellulases show excellent performance in denim finishing, although each enzyme is unique in its own way. When applying the enzyme corresponding to SEQ ID NO: 3 for denim finishing it is possible to reach a high abrasion level with a minimum of strength loss. When treating denim with the enzyme corresponding to SEQ ID NO: 12, a very high wash down can be reached

which leaves the fabric with an almost bleached appearance. Denim finishing with the enzyme corresponding to SEQ ID NO: 8 gives a high abrasion level at a low temperature optimum which makes it possible to reduce the processing temperature and save energy.

J. Use of cloned cellulases from *Acremonium sp.* and *Thielavia terrestris* for Biopolishing of lyocell fibers

Lyocell fibers which are sold under the trade name Tencel are spun from wood pulp cellulose in a more environmentally friendly waterbased solvent than is the case for normal viscose production). However, the fibers have a tendency to fibrillate when they are processed into textiles which is seen on the surface and denoted "fuzz". By using cellulases it is possible to permanently remove the exposed and fuzzy fibers and significantly improve the look of the finished fabric, the treatment generally known as Biopolishing. The endoglucanases of the present invention are especially suited for the removal of Lyocell surface fibers.

MATERIALS AND METHODS

The textile substrate was either 100 % woven or different kinds of jersey knitted dark blue Tencel. The dark colour and jersey knit was preferred in order to enhance the visual effects which simplifed the evaluation. A woven 70/30 Tencel/Rayon blend was also used to a lesser extent.

The assays were either performed in 200 ml scale using a Launder-o-meter or in the 20 l scale using a Wascator. The treatment time was 60 min at 55°C in Wascator and 60-90 min in LOM. The buffer was 2 g/l sodium acetate adjusted to pH 5 with acetic acid. The fabric to liquid ratio was 1:10 but in the Launder-o-meter 20 steel balls with a diameter of 14 mm (11 g each) was used to obtain sufficient mechanical abrasion. The biopolishing was immediately followed by inactivation using 2 g/l sodium carbonate at 80°C for 15 min followed by rinsing in cold water.

The results were evaluated using a fuzz note scale from 1 - 5 were 1 is the fibrillated look of the starting material and 5 is a high quality look with no visible fibers on the surface. Since the performance of endocellulases is specific towards a surface treatment the weightloss is below 2 % and is therefore not included in the evaluation. Two cellulases were evaluated: the cellulases cloned from *Acremonium sp.* (SEQ ID NO: 8) and from *Thielavia terrestris* (SEQ ID NO: 12).

The two cellulases are able to defibrillate both Tencel and Tencel blended fabrics. By using an endoglucanase of the invention, only small fibrils are removed rather than whole fibers such as is the case when using acid cellulase mixtures from *Trichoderma*. The strength loss of the treated fabric is threrefore kept at a minimum when using endoglucanases of the present invention.

The following dosages gave a superior defibrillation, i.e. fuzz note 4 or above:

15 S-CEVU/g fabric of cellulase from *Acremonium sp* (SEQ ID NO: 8); and

10 S-CEVU/g fabric of cellulase from *Thelavia terrestris* (SEQ ID NO: 12).

EXAMPLE 2

A new cellulytic enzyme was by expression cloning as well as by PCR cloning detected to be produced by a plant pathogen, isolated from soy bean seeds and identified as Macrophomina phaseolina.

Production of biomass for PCR and expression cloning procedures:

Isolate CBS 281.96 was grown in shake flask cultures on cellulose enriched potato dextrose broth, incubated for 5 days at 260C (shaking conditions: 150 rpm).

A. Cloning and expression of an endoglucanase from *Macrophomina phaseolina*

mRNA was isolated from *Macrophomina phaseolina*, grown in a cellulose-containing fermentation medium with agitation to ensure sufficient aeration. Mycelia were harvested after 3-5 days' growth, immediately frozen in liquid nitrogen and stored at -80°C. A library from *Macrophomina phaseolina*, consisting of approx. 10^6 individual clones was constructed in *E. coli* as described with a vector background of 1%.

Plasmid DNA from some of the pools was transformed into yeast, and 50-100 plates containing 250-400 yeast colonies were obtained from each pool.

Endoglucanase-positive colonies were identified and isolated on SC-agar plates with the AZCL HE cellulose assay. cDNA inserts were amplified directly from the yeast colonies and characterized as described in the Materials and Methods section above. The DNA sequence of the cDNA encoding the endoglucanase is of SEQ ID NO: 13 and the corresponding amino acid sequence is of SEQ ID NO: 14.

The cDNA is obtainable from the plasmid in DSM 10512.

Total DNA was isolated from a yeast colony and plasmid DNA was rescued by transformation of *E. coli* as described above. In order to express the endoglucanse in *Aspergillus*, the DNA was digested with appropriate restriction enzymes, size fractionated on gel, and a fragment corresponding to the endoglucanase gene was purified. The gene was subsequently ligated to pHD414, digested with appropriate restriction enzymes, resulting in the plasmid pA2C477.

After amplification of the DNA in *E. coli* the plasmid was transformed into *Aspergillus oryzae* as described above.

Screening of the cDNA library by hybridization and characterization of the positive clones.

Approximately 6000 colony forming units (c.f.u.) from the *Macrophomina phaseolina* cDNA library in *E. coli* was screened by colony hybridization using a random-primed ^{32}P-labeled PCR product from *M. phaseolina* as probe. The PCR product was generated as described in the Materials and methods section. The positive cDNA clones were characterized by sequencing the ends of the cDNA inserts, and by determining the nucleotide seuence of the longest cDNA from both strands. The DNA sequence of the cDNA encoding the endoglucanase is of SEQ ID NO: 13 and the corresponding amino acid sequence is of SEQ ID NO: 14.

B. Construction of gene fusion between the endoglucanase from *Macrophomina phaseolina* and the 43 kD endoglucanase from *Humicola insolens*

One construction was prepared in order to make a derivative of the endoglucanase from *M. phaseolina* with the linker and CBD from the 43 kD endoglucanase from *H. insolens* (disclosed in WO 91/17243). The native endoglucanase from *M. phaseolina* does not have a linker and/or a cellulose binding domain, CBD.

The construction consists of the endoglucanase from *M. phaseolina* (223 amino acids) and the 72 C-terminal amino acids from the *H. insolens* 43 kD endoglucanase. (SEQ ID NO: 24)

The 43 kD endoglucanase cDNA from *H. insolens* is cloned into pHD414 in such a way that the endoglucanase gene is transcribed from the Taka-promoter. The resulting plasmid is named pCaHj418.

The cDNA encoding the endoglucanase from *M. phaseolina* (SEQ ID NO: 23) is cloned into pYES2.0 as a BstX I/Not I fragment and the resulting plasmid is named pC1C477.

Primers:

primer 1:

5'- GGTCGCCCGGACTGGCTGTTCCCGTACCCCCTCCAGCAGCACCAGCTCTCCGG -3' (SEQ ID NO: 94)

primer 2:

5'- CCGGAGAGCTGGTGCTGCTGGAGGGGGTACGGGAACAGCCAGTCCGGGCGACC -3' (SEQ ID NO: 95)

pYES2.0 F.HT primer: 5'- CGGACTACTAGCAGCTGTAATACG -3' (SEQ ID NO: 96)

AMG-term. Primer: 5'- CCCCATCCTTTAACTATAGCG -3' (SEQ ID NO: 93)

The endoglucanase fusion is constructed by the PCR overlap-extension method as described by Higuchi et al. 1988.

Reaction A: The Polymerase Chain Reaction (PCR) is used to amplify the fragment of pCaHj418 between primer 1 and AMG-term. primer (the linker and CBD from the 43 kD endoglucanase from *H. insolens*).

Reaction B: PCR amplification of the fragment between pYES2.0 F.HT primer and primer 2 in pC1C477, the endoglucanase gene from *M. phaseolina*.

Reaction C: The two purified fragments are used in a third PCR in the presence of the primers flanking the total region, i.e. pYES2.0 F.HT primer and AMG-term. primer.

The fragment amplified in reaction C is purified, digested with restriction enzymes, e.g. Xba I and BamH I. The purified digested fragment is ligated into pHD414 digested with restriction enzymes, e.g. Xba I and BamH I.

Competent cells from *E. coli* strain DH5αF' (New England Biolabs) are transformed with the ligated plasmid and colonies containing the gene fusion are isolated. The sequence of the cloned part was verified by DNA sequencing.

Polymerase Chain Reactions are carried out under standard conditions, as recommended by Perkin-Elmer.

Reaction A and B start with 2 min. at 94°C followed by 20 cycles of (30 sec. at 94°C, 30 sec. at 52°C and 1 min. at 72°C) and ends with 4 min. at 72°C.

Reaction C starts with (2 min. at 94°C, 1 min. at 52°C and 2 min. at 72°C), followed by 20 cycles of (30 sec. at 94°C, 30 sec. at 52°C and 90 sec. at 72°C) and ends with 4 min. at 72°C.

The construct may be transformed into *Aspergillus oryzae* as described above.

EXAMPLE 3

Cloning and expression of an endoglucanase from *Acremonium sp.* and *Sordaria fimicola*

Production of biomass for expression cloning procedures:

Isolates CBS 478.94 and ATCC 52644, respectively, were grown in shake flask cultures on cellulose enriched potato dextrose broth, incubated for 5 days at 260C (shaking conditions: 150 rpm).

mRNA was isolated from *Acremonium sp.*, CBS 478.94, and *Sordaria fimicola*, ATCC 52644, respectively, grown in a cellulose-containing fermentation medium with agitation to ensure sufficient aeration. Mycelia were harvested after 3-5 days' growth, immediately frozen in liquid nitrogen and stored at -80°C. Libraries from *Acremonium sp.*, and *Sordaria fimicola*, respectively, each consisting of approx. 10^6 individual clones were constructed in *E. coli* as described with a vector background of 1%.

Plasmid DNA from some of the pools from each library was transformed into yeast, and 50-100 plates containing 250-400 yeast colonies were obtained from each pool.

Endoglucanase-positive colonies were identified and isolated on SC-agar plates with the AZCL HE cellulose assay. cDNA inserts were amplified directly from the yeast colonies and characterized as described in the Materials and Methods section above.

The DNA sequence of the cDNA encoding the endoglucanase from *Acremonium sp.* is of SEQ ID NO: 6 and the corresponding amino acid sequence is of SEQ ID NO: 7. The cDNA is obtainable from the plasmid in DSM 10080.

The partial DNA sequence of the cDNA encoding the endoglucanase from *Sordaria fimicola* is of SEQ ID NO: 25 (Nucleotide sequence of the 5'-end of the cDNA) and the corresponding amino acid sequence is of SEQ ID NO: 26. The cDNA is obtainable from the plasmid in DSM 10576.

Total DNA was isolated from a yeast colony and plasmid DNA was rescued by transformation of *E. coli* as described above. In order to express the endoglucanase in *Aspergillus*, the DNA was digested with appropriate restriction enzymes, size fractionated on gel, and a fragment corresponding to the endoglucanase gene from *Acremonium sp.* and *Sordaria fimicola*, respectively, was purified. The genes were subsequently ligated to pHD414, digested with appropriate restriction enzymes, resulting in the plasmids pA2C371 and pA2C502, respectively.

After amplification of the DNA in *E. coli* the plasmids were transformed into *Aspergillus oryzae* as described above.

EXAMPLE 4

A. Cloning by PCR an endoglucanase from *Crinipellis scabella*, CBS 280.96

Isolate CBS 280.96 was grown in static flask cultures, holding wheat bran medium (per flask: 300 g wheat bran added 450 ml salt solution), incubated for 6 days at 26°C. After incubation the wheat bran was extracted with destilled water (300 ml per flask) and the extract tested for endoglucanase activity (0.1% AZCL-HE-Cellulose (megazyme) in 1% agarose (Litex agarose, Medinova). Activity was observed on the plates holding pH of 3.0, 7.0 and 9.5.

mRNA was isolated from Crinipellis scabella grown as describe above. Mycelia were harvested after 3-5 days' growth, immediately frozen in liquid nitrogen and stored at -80°C. A library from *Crinipellis scabella*, consisting of approx. 10^6 individual clones was constructed in *E. coli* as described with a vector background of 1%.

Approximately 10 000 colony forming units (c.f.u.) from the *Crinipellis scabella* cDNA library in *E. coli* was screened by colony hybridization using a random-primed ^{32}P-labeled PCR product from *C. scabella* as probe. The PCR product was generated as described in the Materials and methods section. The positive cDNA clones were characterized by sequencing the ends of the cDNA inserts, and by determining the nucleotide seuence of the longest cDNA from both strands.

The DNA sequence of the cDNA encoding the endoglucanase is of SEQ ID NO: 15 and the corresponding amino acid sequence is of SEQ ID NO: 16.

The cDNA is obtainable from the plasmid in DSM 10511.

Total DNA was isolated from a yeast colony and plasmid DNA was rescued by transformation of *E. coli* as described above. In order to express the endoglucanse in *Aspergillus*, the DNA was digested with appropriate restriction enzymes, size fractionated on gel, and a fragment corresponding to the endoglucanase gene was purified. The gene was subsequently ligated to pHD414, digested with appropriate restriction enzymes, resulting in the plasmid pA2C475.

After amplification of the DNA in *E. coli* the plasmid was transformed into *Aspergillus oryzae* as described above.

Construction of two gene fusions between the endoglucanase from *Crinipellis scabella* and the linker/CBD region of the 43 kDa endoglucanase from *Humicola insolens*.

The native endoglucanase from *Crinipellis scabella* neither has a linker nor a cellulose binding domain (CBD). In addition, the full-length cDNA contains an ATG start codon upstream from the endoglucanase encoding open reading frame (ORF), presumably resulting in scrambled translation initiation upon heterologous expression of the cDNA, such as in the yeast *Saccharomyces cerevisiae* and the filamentous fungus *Aspergillus oryzae*. Thus, two gene fusions between the endoglucanase from *Crinipellis scabella* and the linker/CBD region of the 43 kD endoglucanase from *Humicola insolens* (disclosed in WO 91/17243) has been constructed using splicing by overlap extension (SOE) (Horton et al, 1989).

Construction 1 consists of the cDNA encoding the 226-residue endoglucanase from *C. scabella* fused by PCR with the 3'-end cDNA of *H. insolens* coding for the linker and CBD region (72 amino acids) at the COOH-terminus of the *H. insolens* 43 kD endoglucanase. The second hybrid construct is identical to the abovementioned gene fusion, except that the first five residues from the putative signal peptide have been deleted by PCR resulting in a shorter signal, which starts with the second in-frame ATG start codon.

Plasmid constructs

The plasmid pC1C475 contains the full-length cDNA from *C. scabella*, cloned into BstXI/NotI-cut yeast expression vector pYES 2.0, the plasmid pC1C144 contains the full-length cDNA from *H. insolens*, cloned into the BstXI site of pYES 2.0.

Splicing by overlap extension

Two PCR fragments encoding the core region of the endoglucanase from *C. scabella* were generated in PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatin; containing 200 µM each dNTP), using 50-100 ng of pC1C475 as template, and 250 pmol of the reverse primer (5'-GACCGGAGAGCTGGTGCTGCTGGAGGGTTTACGAACACAGCCCGAGATATTAGTG-3' (SEQ ID NO: 97)) in two combinations with 300-350 pmol of each forward primer (forward no. 1 5'-CCCCAAGCTTGACTTGGAACCAATGGTCCATCC-3' (SEQ ID NO: 98), forward no. 2 5'-CCCCAAGCTTCCATCCAAACATGCTTAAAACGCTCG-3' (SEQ ID NO: 99)), a DNA thermal cycler (Landgraf, Germany) and 2.5 units of Taq polymerase (Perkin-Elmer, Cetus, USA). Thirty cycles of PCR were performed using a cycle profile of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72 □C for 3 min. The PCR fragment coding for the linker and CBD of the endoglucanase of *H. insolens* was generated in PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatin; containing 200 µM each dNTP) using 100 ng of the pC1C144 template, 250 pmol forward primer (5'-CACTAATATCTCGGGCTGTGTTCGTAAACCCTCCAGCAGCACCAGCTCTCCGGTC-3' (SEQ ID NO: 100)), 250 pmol of the pYES 2.0 reverse primer (5'- GGGCGTGAATGTAAGCGTGACATA -3' (SEQ ID NO: 101)), a DNA thermal cycler (Landgraf, Germany) and 2.5 units of Taq polymerase (Perkin-Elmer, USA). Thirty cycles of PCR were performed as above. The PCR products were electrophoresed in 0.7 % low gelling temperature agarose gels (SeaPlaque, FMC), the fragments of interest were excised from the gel and recovered by treatment with agarase (New England Biolabs, USA) according to the manufacturer's instructions, followed by phenol extraction and ethanol precipitation at -20°C for 12 h by adding 2 vols of 96 % EtOH and 0.1 vol of 3 M NaAc.

The recombinant hybrid genes between the endoglucanase from *Crinipellis scabella* and the linker/CBD region of the 43 kD endoglucanase from *Humicola insolens* were generated by combining the overlapping PCR fragments from above (ca. 50 ng of each template) in two combinations in PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatin; containing 200 µM each dNTP). The SOE reaction was carried out using the DNA thermal cycler (Landgraf, Germany) and 2.5 units of Taq polymerase (Perkin-Elmer, Cetus, USA). Two cycles of PCR were performed using a cycle profile of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72 □C for 3 min, the reaction was stopped, 250 pmol of each end-primer (forward no. 1 5'-CCCCAAGCTTGACTTGGAACCAATGGTCCATCC-3' (SEQ ID NO: 98), forward no. 2 5'-CCCCAAGCTTCCATCCAAACATGCTTAAAACGCTCG-3' (SEQ ID NO: 99), reverse primer 5'-GGGCGTGAATGTAAGCGTGACATA-3' (SEQ ID NO: 101)) was added to the reaction

mixture, and an additional 30 cycles of PCR were performed using a cycle profile of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min.

Construction of the expression cassettes for heterologous expression in *Aspergillus oryzae*

The PCR-generated, recombinant fragments were electrophoresed in a 0.7 % low gelling temperature agarose gel (SeaPlaque, FMC), the fragments of interest were excised from the gel and recovered by treatment with agarase (New England Biolabs, USA) according to the manufacturer's instructions, followed by phenol extraction and ethanol precipitation at -20°C for 12 h. The DNA fragments were digested to completion with HindIII and XbaI, and ligated into *Hind*III/*Xba*I-cleaved pHD414 vector followed by electroporation of the constructs into *E. coli* DH10B cells according to the manufacturer's instructions (Life Technologies, USA).

The nucleotide sequence of the resulting gene fusions were determined from both strands as described in the Materials and methods section, SEQ ID NOS: 17 and 19. The constructs may be transformed into *A. oryzae* as described.

EXAMPLE 5

PCR facilitated detection of said type of cellulytic enzyme from 46 filamentous and monocentric fungi, representing 32 genera, from 23 families, belonging to 15 orders of 7 classes, covering all in all all four groups of the true Fungi: Ascomycetous, Basidiomycetous, Chytridiomycetous and Zygomycetous fungi

5.1 Materials

1. *Diplodia gossypina* Cooke

Deposit of Strain, Acc No: CBS 274.96

2. *Ulospora bilgramii* (Hawksw. et al.)

Acc No of strain: NKBC 1444,

3. *Microsphaeropsis sp*

4. *Ascobolus stictoideus* Speg.

Acc No of strain: Q026 (Novo Nordisk collection)

Isolated from goose dung, Svalbard, Norway

5. *Saccobolus dilutellus* (Fuck) Sacc.

Deposit of strain: Acc No CBS 275.96

6. *Penicillium verruculosum* Peyronel

Ex on Acc No of species: ATCC 62396

7. *Penicillium chrysogenum* Thom

Acc No of Strain: ATCC 9480

8. *Thermomyces verrucosus* Pugh et al

Deposit of Strain, Acc No.: CBS 285.96

9. *Xylaria hypoxylon* L. ex Greville

Deposit of Strain, Acc No: CBS 284.96

10. *Poronia punctata* (Fr.ex L.) Fr.

Ref:A.Munk: Danish Pyrenomycetes,

Dansk Botanisk Arkiv, Vol17,1 1957

11. *Nodulisporum sp*

Isolated from leaf of Camellia reticulatá (Theaceae, Guttiferales),

Kunming Botanical Garden, Yunnan Province, China

12. *Cylindrocarpon sp*

Isolated from marine sample, the Bahamas

13. *Fusarium anguioides* Sherbakoff

Acc No of strain: IFO 4467

14. *Fusarium poae* (Peck) Wr.

Ex on Acc No of species: ATCC 60883

15. *Fusarium solani* (Mart.)Sacc.emnd.Snyd & Hans.

Acc No of strain: IMI 107.511

16. *Fusarium oxysporum ssp lycopersici* (Sacc.)Snyd. & Hans.

Acc No of strain: CBS 645.78

17. *Fusarium oxysporum ssp passiflora*

Acc No of strain: CBS 744.79

18. *Gliocladium catenulatum* Gillman & Abbott

Acc. No. of strain: ATCC 10523

19. *Nectria pinea* Dingley

Deposit of Strain, Acc. No. CBS 279.96

20. *Sordaria macrospora* Auerswald

Ex on Acc No of species: ATCC 60255

21. *Humicola grisea* Traeen

ex on Acc No for the species: ATCC 22726

22. *Humicola nigrescens* Omvik

Acc No of strain: CBS 819.73

23. Scytalidium thermophilum (Cooney et Emerson) Austwick

Acc No of strain: ATCC 28085

24. *Thielavia thermophila* Fergus et Sinden

(syn *Corynascus thermophilus*)

Acc No of strain: CBS 174.70, IMI 145.136

25. *Cladorrhinum foecundissimum* Saccardo et Marchal

Ex on Acc No of species: ATCC 62373

26. *Syspastospora boninensis*

Acc No of strain: NKBC 1515 (Nippon University, profe Tubaki Collection)

27. *Chaetomium cuniculorum* Fuckel

Acc. No. of strain: CBS 799.83

28. *Chaetomium brasiliense* Batista et Potual

Acc No of strain: CBS 122.65

29. *Chaetomium murorum* Corda

Acc No of strain: CBS 163.52

30. *Chaetomium virescens* (von Arx) Udagawa

Acc.No. of strain: CBS 547.75

31. *Nigrospora sp*

Deposit of strain, Acc No: CBS 272.96

32. *Nigrospora sp*

Isolated from:

33. *Diaporthe syngenesia*

Deposit of strain, Acc No: CBS 278.96

34. *Colletotrichum lagenarium* (Passerini) Ellis et Halsted

syn *Glomerella cingulata* var orbiculare Jenkins et Winstead

Ex on acc No of species: ATCC 52609

35. *Exidia glandulosa* Fr.

Deposit of Strain, Acc No: CBS 277.96

36. *Fomes fomentarius* (L.) Fr.

Deposit of strain: Acc No. CBS 276.96

37. *Spongipellis* (?)

Deposit of Strain: Acc No CBS 283.96

38. *Rhizophlyctis rosea* (de Bary & Wor) Fischer

Deposit of Strain: Acc No.: CBS 282.96

39. Rhizomucor pusillus (Lindt) Schipper

syn: *Mucor pusillus*

Acc No of strain: IFO 4578

40. *Phycomyces nitens* (Kunze) van Tieghem & Le Monnier

Acc No of strain: IFO 4814

41. *Chaetostylum fresenii* van Tieghem & Le Monnier

syn. *Helicostylum fresenii*

Acc No of strain NRRL 2305

42. *Trichothecium roseum*, Acc No of strain: IFO 5372

43. *Coniothecium sp*

Endophyte, isolated from leaf of flowering plant,

Kunming, Yunnan, China

44. Deposit of strain, Acc No.: CBS 271.96

Coelomycete, Isolated from leaf of *Artocarpus altilis*

(Moraceae, Urticales), Christiana, Jamaica

45. Deposit of strain, Acc No.: CBS 273.96

Coelomycete, isolated from leaf of *Pimenta dioica*

(Myrtaceae, Myrtales), Dallas Mountain, Jamaica

46. Deposit of strain: CBS 270.96

Coelomycete, isolated from leaf of *Pseudocalymma alliaceum* (Bignoniaceae, Solanales) growing in Dallas Mountain, Jamaica

5.2 Procedure

Maintenance of strains and production of biomass:

The strains were maintained on agar in petrie dishes (9 cm) or on slants (see list of Media: PCA and PDA). 44 of the strains were grown in shake flasks under the following growth conditions: general fungal media as PC, PD and PB 9 or YPG (see list of media); incubation time from 3 to 9 days; temperature 26°C; rpm between 150 and 175. Strain No 14 (*F. poae*) was grown on wheat bran for 15 days (26°C; static). Strain No 38 was grown in dilute salt solution (DS/2), added 1 cm^2 pieces of autoclaved filter paper.

Activity test:

Activity was tested on 0.1% AZCL-HE-Cellulose (Megazyme) plates (14 cm Petrie dishes), made up in 1% agarose (HSB, Litex Agarose, Medinova). All tests were done in triplicate, viz. AZCL-HE-Cellulose dissolved in three buffers, adjusted to pH 3, 7 or 9.5 (using various proportions

of the following two ingredients Citric acid monohydrat, Merck art. No 100244 (21.0 g) dissolved in water, making a total of 1000 ml; 0.1M tri-Sodium dodecabrohydrate, Merck art.no. 6578 (38 g), dissolved in water, making a total of 1000 ml. The mixing is done immidiately before use.

Harvesting of Biomass:

The biomass was harvested by filtering (mesh adjusted to the growth of the fungus, the finest used for fungi which have highly sporulating mycelium as e.g. Fusarium spp.). The biomass on the filter was scraped into a sterile plastic bag and immidiately frozen (by submerging into liquid nitrogen).

5.3 Results

I. Using the PCR screening and amplification techniques described in Materials and Methods the following partial cDNA sequences were obtained:

Saccobolus dilutellus (Fuck) Sacc., CBS 275.96: SEQ ID NO: 27 (and the deduced amino acid sequence in SEQ ID NO: 28);

Thermomyces verrucosus, CBS 285.96: SEQ ID NO: 29 (and the deduced amino acid sequence in SEQ ID NO: 30);

Xylaria hypoxylon, CBS 284.96: SEQ ID NO: 31 (and the deduced amino acid sequence in SEQ ID NO: 32);

Fusarium oxysporum ssp lycopersici, CBS 645.78: SEQ ID NO: 33 (and the deduced amino acid sequence in SEQ ID NO: 34);

Nectria pinea, CBS 279.96: SEQ ID NO: 35 (and the deduced amino acid sequence in SEQ ID NO: 36);

Humicola grisea, ATCC 22726: SEQ ID NO: 37 (and the deduced amino acid sequence in SEQ ID NO: 38);

Humicola nigrescens, CBS 819.73: SEQ ID NO: 39 (and the deduced amino acid sequence in SEQ ID NO: 40);

Cladorrhinum foecundissimum, ATCC 62373: SEQ ID NO: 41 (and the deduced amino acid sequence in SEQ ID NO: 42);

Syspastospora boninensis, NKBC 1515: SEQ ID NO: 43 (and the deduced amino acid sequence in SEQ ID NO: 44);

Nigrospora sp., CBS 272.96: SEQ ID NO: 45 (and the deduced amino acid sequence in SEQ ID NO: 46);

Chaetostylum fresenii: SEQ ID NO: 47 (and the deduced amino acid sequence in SEQ ID NO: 48);

Exidia glandulosa, CBS 277.96: SEQ ID NO: 49 (and the deduced amino acid sequence in SEQ ID NO: 50);

Coniothecium sp.: SEQ ID NO: 51 (and the deduced amino acid sequence in SEQ ID NO: 52);

Deposition No. CBS 271.96: SEQ ID NO: 53 (and the deduced amino acid sequence in SEQ ID NO: 54);

Deposition No. CBS 270.96: SEQ ID NO: 55 (and the deduced amino acid sequence in SEQ ID NO: 56);

Diplodia gossypina, CBS 274.96: SEQ ID NO: 57 (and the deduced amino acid sequence in SEQ ID NO: 58);

Ulospora bilgramii, NKBC 1444: SEQ ID NO: 59 (and the deduced amino acid sequence in SEQ ID NO: 60);

Penicillium verruculosum, ATCC 62396: SEQ ID NO: 61 (and the deduced amino acid sequence in SEQ ID NO: 62);

Poronia punctata: SEQ ID NO: 63 (and the deduced amino acid sequence in SEQ ID NO: 64);

Fusarium anguioides, IFO 4467: SEQ ID NO: 65 (and the deduced amino acid sequence in SEQ ID NO: 66);

Thielavia thermophila, CBS 174.70: SEQ ID NO: 67 (and the deduced amino acid sequence in SEQ ID NO: 68);

Chaetomium cuniculorum, CBS 799.83: SEQ ID NO: 69 (and the deduced amino acid sequence in SEQ ID NO: 70);

Chaetomium virescens: SEQ ID NO: 71 (and the deduced amino acid sequence in SEQ ID NO: 72);

Colletotrichum lagenarium: SEQ ID NO: 73 (and the deduced amino acid sequence in SEQ ID NO: 74);

Phycomyces nitens: SEQ ID NO: 75 (and the deduced amino acid sequence in SEQ ID NO: 76); and

Trichothecium roseum: SEQ ID NO: 77 (and the deduced amino acid sequence in SEQ ID NO: 78);

II. Using the PCR screening and amplification techniques described in Materials and Methods partial cDNA encoding partially for the enzyme of the invention was obtained and the plasmid was deposited according to the Budapest Treaty:

Escherichia coli, DSM 10583, deposition date 13 March, 1996;
cDNA from *Trichothecium roseum*;
Escherichia coli, DSM 10584, deposition date 13 March, 1996;
cDNA from *Syspastospora boninensis*;
Escherichia coli, DSM 10585, deposition date 13 March, 1996;
cDNA from *Cheatomium murorum*;
Escherichia coli, DSM 10587, deposition date 13 March, 1996;
cDNA from *Sordaria fimicola*;
Escherichia coli, DSM 10588, deposition date 13 March, 1996;
cDNA from the unidentified strain CBS 273.96;
Escherichia coli, DSM 10586, deposition date 13 March, 1996;
cDNA from *Spongipellis sp.*

Color clarification of crude supernatants

During normal wash the fabric will often fade. However, the fabric appearance is improved and the original colours are much better preserved or maintained if the fabric is washed with a cellulase giving color clarification. Color clarification is measured as removal of surface fibrils and fibers protruding from the yarn of a textile containing cellulosic fibers.

Apparatus	:	Terg-o-tometer
Liquid volume	:	100 ml
Agitation	:	150 movements/min with vertical stirrer
Rinse time	:	5 min in tapwater
Washing temp	:	40°
Washing liqour	:	0.05 M phosphate buffer
pH	:	7.0
Washing time	:	30 min
Repetitions	:	2
Enzymes	:	Crude supernatants from the strains shown below.
Dosage	:	Two dosages from 200, 500, 1000 or 2500 S-CEVU/l
Textile	:	2 swatches of aged black 100% cotton 5x6 cm (0.9 gram)
Drying	:	Tumble dry

Evaluation:

The light remission is measured by a Datacolor Elrepho Remission spectrophotometer. Remission is calculated as delta L (Hunter Lab-values). When the surface fibrils and fibers protruding from the yarn are removed by the cellulase, the surface of the black fabric appears darker, and lower L values are obtained.

The samples are compared with a blind sample, i.e. washed without enzyme. Below is shown the delta L remission values compared to a blind sample.

REFERENCES

Background of the invention:

1. GB-A-1368599
2. EP-A-0 307 564
3. EP-A-0 435 876
4. WO 91/17243
5. WO 91/10732
6. WO 91/17244
7. WO 95/24471
8. WO 95/26398
9. Methods in Enzymology, 1988, Vol. 160, p. 200-391 (edited by Wood, W.A. and Kellogg, S.T.).
10. Béguin, P., "Molecular Biology of Cellulose Degradation", Annu. Rev. Microbiol. (1990), Vol. 44, pp. 219-248.
11. Henrissat, B., "Cellulases and their interaction with cellulose", Cellulose (1994), Vol. 1, pp. 169-196.
12. T.-M. Enveri, "Microbial Cellulases" in W.M. Fogarty, Microbial Enzymes and Biotechnology, Applied Science Publishers, 183-224 (1983).
13. Béguin, P. and Aubert, J-P., "The biological degradation of cellulose", FEMS Microbiology Reviews 13 (1994) 25-58.
14. Sheppard, P.O., et al., "The use of conserved cellulase family-specific sequences to clone Cellulase homologue cDNAs from *Fusarium oxysporum*, Gene, (1994), Vol. 15, pp. 163-167.
15. Saloheimo, A., et al., "A novel, small endoglucnaase gene, *egl5*, from *Trichoderma reesei* isolated by expression in yeast", Molecular Microbiology (1994), Vol. 13(2), pp. 219-228.
16. van Arsdell, J.N. et al., (1987) Cloning, characterization, and expression in *Saccharomyces cerevisiae* of endoglucanase I from *Trichoderma reesei*, Bio/Technology **5**: 60-64.

17. Penttilä, M. et al, (1986) Homology between cellulase gnees of *Trichoderma reesei*: complete nucleotide sequence of the endoglucanase I gene. *Gene* **45**:253-263.

18. Saloheimo, M. et al, (1988) EGIII, a new endoglucanase from *Trichoderma reesei*: the characterization of both gene and enzyme. *Gene* **63**:11-21.

19. Gonzáles, R., et al., "Cloning, sequence analysis and yeast expression of the *egl1* gene from *Trichoderma longibrachiatum*", Appl. Microbiol. Biotechnol. (1992), Vol. 38, pp. 370-375.

20. Ooi, T. et al. "Cloning and sequence analysis of a cDNA for cellulase (FI-CMCase) from *Aspergillus aculeatus*" Curr. Genet. (1990), Vol. 18, pp. 217-222.

21. Ooi, T. et al, "Complete nucleotide sequence of a gene coding for *Aspergillus aculeatus* cellulase (FI-CMCase)" Nucleic Acids Research (1990), Vol. 18, No. 19, p. 5884.

22. Xue, G. et al., "Cloning and expression of multiple cellulase cDNAs fromthe anaerobic rumen fungus *Neocallimastix patriciarum* in *E. coli*, J. Gen. Microbiol. (1992), Vol. 138, pp. 1413-1420.

23. Xue, G. et al., "A novel polysaccharide hydrolase cDNA (*celD*) from *Neocallimastix patriciarum* encoding three multi-functional catalytical domains with high endoglucanase, cellobiohydrolase and xylanase activities", J. Gen. Microbiol. (1992), Vol. 138, pp. 2397-2403.

24. Zhou, L. et al., "Intronless *celB* from the anaerobic fungus *Neocallimastix patriciarum* encodes a modular family A endoglucanase", Biochem. J. (1994), Vol. 297, pp. 359-364.

25. Dalbøge, H. and Heldt-Hansen, H.P., "A novel method for efficient expression cloning of fungal enzyme genes", Mol. Gen. Genet. (1994), Vol. 243, pp. 253-260.

26. Ali, B.R.S. et al., "Cellulases and hemicellulases of the anaerobic fungus *Piromyces* constitute a multiprotein cellulose-binding complex and are encoded by multigene families", FEMS Microbiol. Lett. (1995), Vol. 125, No. 1, pp. 15-21.

27. DNA Data Bank of Japan (DDBJ).

28. Wang, H.Y. and Jones, R.W.: "Cloning, characterization and functional expression of an endoglucanase-encoding gene from the phytopathogenic fungus *Macrophomina phaseolina*", Gene, 158:125-128, 1995.

29. Wang, H.Y. and Jones, R.W.: "A unique endoglucanase-encoding gene cloned from the phytopathogenic fungus *Macrophomina phaseolina*", Appl. and Environm. Microbiology, 61:2004-2006, 1995.

30. B. Henrissat: Biochem. J., **280**:309-316, 1991.

31. Schauwecker, F., Wanner, G., Kahmann, R.: "Filament-specific expression of a cellulase gene in the dimorphic fungus Ustilago maydis", 1995, Biological Chemistry Hoppe-Seyler, 376:617-625.

32. WO 93/20193

33. WO 94/21801

34. WO 94/26880

35. WO 95/02043

The Drawings:

1. Feng and Doolittle, 1987, J. Mol. Evol. 25: 351-360.

2. NIH Data Base (Entrez, version spring 1996) available on World Wide Web: (http://www3.ncbi.nlm.nih.gov/htbin/ef/entrezTAX).

3. Eriksson, O.E. & Hawksworth, D.L.: Systema Ascomycetum vol 12 (1993).

4. Jülich, W.: Higher Taxa of Basidiomycetes, Bibliotheca Mycologia 85, 485 pp (1981).

5. O´Donnell, K.: Zygomycetes in culture, University of Georgia, US, 257 pp (1979).

6. Hawksworth, D.L., Kirk, P.M., Sutton, B.C. and Pegler, D.N.: Dictionary of the fungi, International Mycological Institute, 616 pp (1995).

7. Von Arx, J.A.: The genera of fungi sporulating in culture, 424 pp (1981).

Detailed Description:

1. Ford et al., Protein Expression and Purification 2: 95-107, 1991.

2. Cunningham and Wells, Science 244, 1081-1085, 1989.

3. de Vos et al., Science 255: 306-312, 1992.

4. Smith et al., J. Mol. Biol. 224: 899-904, 1992.

5. Wlodaver et al., FEBS Lett. 309: 59-64, 1992.

6. Tomme, P. et al. "Cellulose-Binding Domains: Classification and Properties" in "Enzymatic Degradation of Insoluble Carbohydrates", John N. Saddler and Michael H. Penner (Eds.), ACS Symposium Series, No. 618, 1996.

7. WO 90/00609

8. WO 95/16782

9. Needleman, S.B. and Wunsch, C.D., *Journal of Molecular Biology*, 48: 443-453, 1970.

10. WO 94/14953

11. Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab., Cold Spring Harbor, NY.

12. Beaucage and Caruthers, Tetrahedron Letters 22 (1981), 1859 - 1869.

13. Matthes et al., EMBO Journal 3 (1984), 801 - 805.

14. US 4,683,202

15. Saiki et al., Science 239 (1988), 487 - 491.

16. Hitzeman et al., J. Biol. Chem. 255 (1980), 12073 - 12080.

17. Alber and Kawasaki, J. Mol. Appl. Gen. 1 (1982), 419 - 434).
18. Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York, 1982).
19. US 4,599,311
20. Russell et al., Nature 304 (1983), 652 - 654.
21. McKnight et al., The EMBO J. 4 (1985), 2093 - 2099
22. P.R. Russell, Gene 40, 1985, pp. 125-130.
23. US 4,870,008
24. O. Hagenbuchle et al., Nature 289, 1981, pp. 643-646.
25. L.A. Valls et al., Cell 48, 1987, pp. 887-897.
26. WO 87/02670
27. M. Egel-Mitani et al., Yeast 6, 1990, pp. 127-137.
28. US 4,546,082
29. EP 16 201
30. EP 123 294
31. EP 123 544
32. EP 163 529
33. WO 89/02463
34. WO 92/11378
35. US 4,599,311
36. US 4,931,373
37. US 4,870,008
38. US 5,037,743
39. US 4,845,075
40. US 4,931,373
41. Gleeson et al., J. Gen. Microbiol. 132, 1986, pp. 3459-3465.
42. US 4,882,279
43. EP 272 277
44. EP 230 023
45. Malardier et al., 1989, Gene 78: 147-156.
46. WO 93/11249.
47. WO 94/14953.
48. WO 95/02043.
49. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989) *Gene* **77,** 61-68

50. Dalbøge, H., and Heldt-Hansen, H. (1994) *Mol. Gen. Genet.* **243,** 253-260

51. Christensen, T., Wøldike, H., Boel, E., Mortensen, S. B., Hjortshøj, K., Thim, L., and Hansen, M. T. (1988) *Bio/Technology* **6,** 1419-1422

52. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463-5467

53. Devereux, J., Haeberli, P., and Smithies, O. (1984) *Nucleic Acids Res.* **12,** 387-395

54. Becker, D. M. & Guarante, L. 1991. Methods Enzymol. 194: 182-187.

55. Gubler, U. & Hoffman, B. J. 1983. Gene 25: 263-269.

56. R. Higuchi, B. Krummel, and R.K. Saiki (1988). A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucl. Acids Res. 16: 7351-7367.

57. Sanger, F., Nicklen, S. & Coulson, A. R. 1977. Proc. Natl. Acad. Sci. U. S. A. 74: 5463-5467.

58. N. Axelsen et al., A Manual of Quantitative Immunoelectrophoresis, Blackwell Scientific Publications, 1973, Chapters 2,3,4 and 23.